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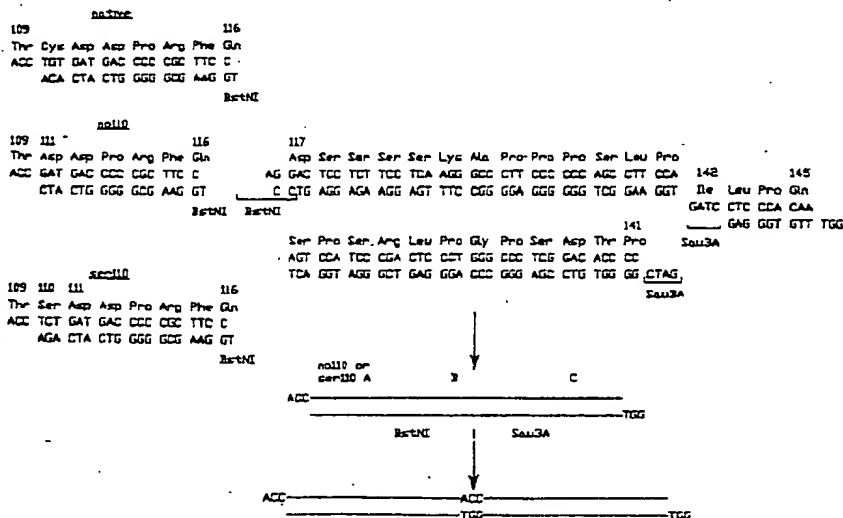
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(54) Title: AUTOANTIGEN VACCINES

(57) Abstract

Autoantigen vaccines are obtained by conferring antigenicity by formation of multimers, of fusion proteins to non-bacterial sequences, or both. The vaccines can be administered using conventional dosage forms for peptide vaccines, or can be supplied in a recombinant vaccinia and the peptides synthesized *in situ*. Particularly useful autoantigens are those derived from hormones controlling reproduction. An easily administered and reversible vaccine for preventing human pregnancy is disclosed. The vaccine comprises a vaccinia virus vector modified to contain the DNA sequence encoding the C-termi-

nal portion (CTP) of the β -chain of human chorionic gonadotropin, or a multimer thereof. The vaccinia vectors are designed so that the encoded antigen is expressed internally by infected cells, or the CTP-encoding sequence is modified to provide a chimera which is capable of carrying the CTP_n antigen to the surface of the infected cell and either presenting the CTP_n antigen at the cell surface or of secreting it. In a preferred embodiment, the influenza hemagglutinin protein, or portions thereof, are used as the components of the chimera. Similar constructions which include alternate hormone derived sequences are also effective in humans and in other mammals. The antibodies obtained against and specific for these autoantigens are also useful.



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-1-

AUTOANTIGEN VACCINES

5

Technical Field

10 This invention relates to construction and
design of vaccines which neutralize the effects of
indigenous proteins. Such vaccines can be made species
specific or can have cross-species effectiveness. They
are particularly useful in the control of fertility in
15 both humans and animals. An important embodiment of
such vaccines utilizes vaccinia-borne antigens.

Background Art

20 Control of population is a long-standing
problem of humankind. It has even been suggested that
the Trojan War was a device of the Zeus to thin out the
densely populated eastern Mediterranean region (Tuchman,
B., The March of Folly (1984), p 47; citing Cypria and
Euripides). More recent efforts have relied on
25 techniques of slightly more sophistication. While
methods which require repeated self-administration have
been moderately successful in some circumstances, many
individuals, and a few countries as a matter of policy,
have found it necessary to rely on long-term approaches
30 such as sterilization or after-the-fact remedies, such
as abortion. Neither of these seems optimal.

Control of fertility in the animal population,
especially among pets, is also a serious problem, at
least in the United States. Millions of unwanted

-2-

puppies and kittens are destroyed annually due to the failure of the owners of animals adequately to prevent reproduction of their pets. In part, this failure results from the inconvenience and expense of the
5 currently available methods of sterilization, which involve surgical procedures. In addition, there appears to be a psychological reluctance on the part of some pet owners to subject their animals to what they perceive as a drastic procedure. While owners of farm animals are
10 not comparably squeamish, it may still be desirable to offer an alternative method of population and behavior control to castration. For example, it is known that castration of bulls at an early age results in
15 diminution in the quantity of meat, and in a higher fat content. However, alternative means for population and behavior control among pets or domesticated farm animals have, so far, been unavailable.

The general concept of the use of vaccines for the control of fertility in both humans and animals has
20 been the subject of some considerable research. In the case of humans, for example, it has been suggested to immunize females against sperm (Li, T.S., Obstet Gynec (1974) 44:607-623) and also against placental and trophoblastic antigens, which in early work utilized
25 fairly crude extracts (Koren, Z., et al, M J Obstet Gynec (1968) 102:340-346; Beck, J.S., et al, J Path (1970) 125-129), and more recently against purer antigens such as human placental lactogen and human chorionic gonadotropin (Stevens, V.C., et al, Am J Repro
30 Immunol (1981) 1:307-314). See also US Patents 4,384,995; 4,526,716; 4,302,386; and 4,201,770; all to Stevens. Synthetic analogs for human chorionic gonadotropin (hCG) have also been suggested as antigens (Matsuura, S., et al, Endocrinol (1979) 101:396-401). A

-3-

recent review of immunological methods has been prepared by Stevens, V.C., in Endocrine Mechanisms in Fertility Regulation, Benagiano, G., and Diczfalusy, E., eds., New York: Raven Press, 1983, pp. 141-162, incorporated

5 herein by reference. This review includes the suggestion that if properly constructed, vaccines having the carboxy terminal peptide of hCG as antigenic component may be useful in humans. In addition, Thau, R.B., et al have, in several papers, referenced below,
10 suggested that ovine luteinizing hormone (oLH) may be useful in immunizing human females against pregnancy.

One of the difficulties in administration of antigens against pregnancy is in finding a suitable vehicle which is acceptable for injection into humans.

15 Ordinary adjuvants, such as Freund's complete adjuvant or Freund's incomplete adjuvant are not acceptable in humans (Stevens, V.C., Serono Symposium No. 45, Academic Press, London & New York, (1982), pp. 131-143). Cost factors in preparing a suitable subunit vaccine have
20 also been recognized as a problem in preparing anti-fertility vaccines for use in less-developed countries. This is particularly poignant because it is in these areas that overpopulation threatens to become a major detriment to further development.

25 With respect to animals, some studies have been made which would be relevant to generating a peptide- or protein-based vaccine to prevent estrus or to destroy male sexual function in animals. Talwar, G.P., et al (Proc Natl Acad Sci (USA) (1985) 82:1228-1231) prepared
30 monoclonal antibodies against gonadotropin releasing hormone (GnRH), which antibodies were capable, when injected into female dogs, of immediately suppressing the progression of estrus. Fraser, H.M., et al (J Endocr (1974) 63:399-406) had, a decade earlier,

-4-

demonstrated that atrophy of the primary and secondary sex characteristics of male rats could be induced by immunization with GnRH (then called LHRH) conjugated to bovine serum albumin. The conjugate presumably raised
5 antibodies to GnRH, which interrupted the normal maintenance of sexual function by neutralizing this hormone. Schanbacher, B.D. (J Androl (1983) 4:233-239) later performed a similar study with similar results using male dogs as subjects. Esbenshade, US Patent
10 4,556,555 disclosed a process for passive neutering of animals by pre-puberty injection of anti-GnRH antiserum.

However, the development of a vaccine to control population and behavior in household pets and farm animals has, apparently, not been seriously
15 undertaken. Furthermore, vaccines prepared from proteins or peptide conjugates have certain inherent disadvantages. Preparation of the peptides and formulation of the vaccines are expensive, and administration must be done under controlled conditions
20 by trained personnel. Also, because these vaccines are prepared by conjugation of the autoantigen to a carrier at the protein, rather than at the genetic level, some denaturation inevitably occurs. It is thus difficult to achieve uniform compositions from batch to batch, and
25 some compositions have lowered antigenicity, resulting in variable immune responses.

An alternative to protein-based vaccines has been known for many years. Smallpox has been successfully eradicated through the use of vaccines
30 comprised of vaccinia virus. Vaccine comprised of vaccinia virus has tremendous advantages in terms of cost (approximately two cents per dose) and also in terms of handling, as it can be administered as a scratch under nonsterile field conditions and maintains

-5-

its stability at room temperature when previously freeze-dried. These properties of vaccinia have recently been used to advantage in preparing vaccines against other infectious agents by recombining the genes
5 encoding the desired immunogens against, for example, hepatitis B, herpes, influenza, and rabies, into the vaccinia genome. The recombined vaccinia can then be used in a manner analogous to the original smallpox vaccine, with all the attendant advantages. See, for
10 example, Smith, G.L., and Moss, B., Biotechniques (1984) 306-312; Smith, G.L., Nature (1983) 302:490-495; Panicali, D., et al, Proc Natl Acad Sci (USA) (1983) 80:5364-5368; Paoletti, E., et al, Proc Natl Acad Sci (1984), 81:193-197; Wiktor, T.J., Proc Natl Acad Sci
15 (1984) 81:7194-7198; and Panicali, D., et al, Proc Natl Acad Sci (1982) 79:4927-4931.

The desirable properties of the vaccinia carrier have, however, never been marshalled to administer effective vaccines directed against
20 autoantigens. The appropriate autoantigens for control of fertility depend on the species of the subject, but are generally related to those hormones which regulate the reproductive system. Human chorionic gonadotropin is a particular such antigen which, if neutralized,
25 would be incapable of its necessary role in the maintenance of human pregnancy. Thus, raising neutralizing antibodies against this factor, for example through the vehicle of vaccinia-borne administration would result in effective control of the consequences of
30 conception in humans. The use of chorionic gonadotropin carboxy-terminal peptide is not workable in animals in general, however, because there is no placental production of chorionic gonadotropin in most species except horses. Therefore, for vaccines effective in

-6-

non-human species to achieve sterilization or temporary infertility, alternative autoantigens must be used. DNA sequences encoding a number of hormones known to be significant in controlling the estrus cycle, such as luteinizing hormone (LH) and follicle stimulating hormone (FSH) are usable. However, in a particularly preferred embodiment, the DNA encoding GnRH is effective in generating antibodies which prevent fertility in these animals. As the sexual function of both male and female farm animals and in dogs and cats is controlled primarily by GnRH, either sex may be rendered infertile or sterilized using such vaccines.

Disclosure of the Invention

The invention provides vaccines which are low-cost, effective preparations suitable for use in developing countries as well as in the industrialized world for fertility control, both in humans and in animals. The vaccines are also useful, in general, to control any metabolic function which is regulated by an indigenous protein or autoantigen.

The vaccines include those which provide protection against pregnancy in humans for limited time periods and which are reversible so as to permit their use without the negative effect and disincentive of permanent sterilization. Such vaccines are immunogenic forms of human chorionic gonadotropin (hCG), a placental product necessary for maintenance of pregnancy. Other hormones connected with reproductive function, such as GnRH, and certain forms of LH can also be used as the basis for providing antigenic determinants in the human vaccine. Analogous vaccines using autoantigenic forms of, for example, GnRH, FSH, or LH are useful in fertility control in animals. The immunogens can be

-7-

administered as DNA constructs with vaccinia virus, a vehicle which permits low-cost formulation as well as administration under a range of conditions some of which may be less than desirable for delivery of medical services. They can also be administered in more conventional methods such as protein vaccines using, for example, an autoantigenic construction encoding the appropriate region of the appropriate hormone as the antigenic determinant.

10 The invention, however, has broader application. Indigenous proteins in general can be made effective as autoantigens by multimerization of their epitopes or by conjugation to a non-bacterial amino acid sequence in the form of a fusion protein and can be
15 administered in conventional or vaccinia-borne forms.

Thus, any metabolic function regulated by peptides is susceptible to control by appropriate vaccines. The species specificity of such vaccines can be controlled since this depends upon the choice of epitopes. The
20 vaccines can thus be constructed to be broadly applicable across species lines, or can be limited in their effect to a single species of choice.

In its broadest aspect, the invention relates to the use of autoantigens as vaccines to control
25 metabolic function wherein the antibodies to an appropriate indigenous protein are raised in response to a recombinantly created "autoantigenic" form, such as, preferably, a multimer which contains at least 2 repeating units of at least one epitope associated with
30 the indigenous protein, or as a recombinantly produced fusion protein bearing a suitable epitope. The autoantigenic forms are constructed at the DNA level, using recombinant techniques, and the autoantigenic proteins may either be generated in situ in the subject, if the DNA encoding the autoantigen is properly disposed

-8-

in a vaccinia vehicle, or they may be produced using recombinant techniques and supplied as peptides or proteins.

In one approach, the autoantigen is a multimer, and the DNA encoding an indigenous peptide, such as a hormone, or an epitope-containing portion thereof, is manipulated in vitro to obtain repeating coding sequence for a multimer of this sequence. The proteins which form the basis for the vaccine may also be monomers with respect to an autoantigen epitope if they are disposed in suitable carrier proteins which provide sufficient immunogenicity. The DNA encoding this epitope is, in this case, ligated to the DNA encoding the carrier. Finally, the epitope-containing moiety can be linked to a carrier to create immunogenicity at the protein level.

In one particular aspect, the invention relates to antipregnancy vaccines useful in humans which are based on epitopes of a specific region of the hCG protein - the carboxy terminal peptide (CTP) which represents the end of the "B-hCG" chain. (As will be explained below, hCG contains two non-covalently bound subunits, designated α and β .) These monomers and multimers include CTPs modified by containing no cysteine at position 110 or having cysteine replaced with a serine or other equivalent amino acid residue at position 110. In another embodiment, vaccines against pregnancy in farm animals and pets, and, if properly administered, in humans, are based on the production of immunogenic forms of a fertility controlling autoantigen gonadotropin releasing factor (GnRH) a product of the hypothalamus which controls release of certain hormones from the pituitary. The most preferred forms of GnRH are modifications of the human sequence wherein the N-terminal pyroglutamic acid (presumably formed by

cyclization in serum) is replaced by glutamine and the C-terminal amidated glycine is replaced by glutamine. Still other embodiments utilize immunogenic forms of other fertility controlling autoantigens, specifically, LH and FSH, in both animals and humans.

Also an aspect of the invention are vaccines whose species specificity is regulated by the choice of epitopes in converting portions of human, bovine, and dog LH β chains to autoantigens. Depending on the portions of the peptides chosen, these vaccines are species-specific or species-versatile in their application.

In all cases, the desired autoantigen can if desired be administered in the form of a recombinant vaccinia containing expressible monomers and multimers of the autoantigen. For vectors whose DNA sequence encodes only the autoantigen (for example, the multimers of CTP or GnRH), intracellular expression is expected to result. These antigens will be revealed to the immune system when the infected cells in which they reside are lysed by the cytotoxic immune response mounted against vaccinia. For those embodiments wherein the vaccinia genome is recombined with a carrier protein encoding sequence (such as influenza hemagglutinin (HA) encoding DNA) which is interrupted by the DNA sequences encoding the desired autoantigen, the secondary carrier functions to transport the autoantigen monomers or multimers to the surface of infected cells. This creates more effective exposure of the autoantigen to the subject, and enhances the immune response. In one embodiment, using HA, for example, the desired autoantigen genes are cloned into the A or B antigenic regions of the influenza HA; in a second embodiment, they are cloned between the HA signal sequence and membrane anchor

-10-

portions. Both of these approaches result in presentation of the antigen at the surface of the cell. Other constructions result in the secretion of the expressed protein. For instance, the desired
5 autoantigen DNAs can be cloned into HA encoding DNA so that the resulting proteins are linked operably to the signal sequence, but with the membrane anchor portion of the HA eliminated. Alternatively, other signal
10 sequences, such as, for example, those associated with bacterial alkaline phosphatase (BAP) or renin can be used to effect secretion of the the desired autoantigen immunogens, or the signal sequence associated with the native antigenic protein may be employed.

In other aspects, the invention relates to
15 methods of preventing pregnancy or controlling other metabolic functions by administration of the foregoing vaccines and to methods of preparation of such vaccines, as well as to intermediate vectors and DNA sequences useful in their preparation.

20 In still another aspect, the invention relates to antibodies produced in response to these vaccines. These antibodies are particularly useful in assessing the levels of the hormones themselves through immunoassay. Accordingly also included within the
25 invention are labeled and unlabelled forms of the these antibodies, and these antibodies both in soluble form and bound to a solid support. Spleens obtained from animals immunized with the vaccines of the invention can also be used as a source of cells for immortalization to
30 obtain corresponding monoclonal preparations.

Brief Description of the Drawings

Figure 1 shows the DNA sequences useful in preparing the gene encoding modified and unmodified

-11-

8-hCG CTP monomers and multimers representing amino acids 109-145 of 8-hCG.

Figure 2 shows the amino acid sequence for GnRH in several species and the DNA sequences useful in preparing the genes encoding modified human and chickenII GnRH monomers and multimers.

Figure 3a shows the cDNA sequence of and method of constructing appropriate multimers of a portion of the 8 peptide of bovine LH.

Figure 3b shows the cDNA sequence of and method of constructing appropriate multimers of a portion of the 8 peptide of dog LH.

Figure 4 shows the cDNA sequence of and method of constructing appropriate multimers of a portion of the 8 peptide of human LH.

Figure 5 shows modification of pKK223-3 to obtain the host vector pVA1 and of pKT19 and pKT41 to obtain the host vector pVA2.

Figure 6 shows the construction of derivative vectors pHA1 and pHA2 containing an HA-encoding DNA.

Figure 7 shows a map of a typical hemagglutinin encoding gene with useful restriction sites for its use as a carrier.

Figure 8 shows the modification of the HA gene for insertion of autoantigen multimers in the HA antigenic regions.

Figure 9 shows the construction of intermediate vectors carrying an HA gene modified by insertion of autoantigen between its signal sequence and membrane anchor.

Figures 10a, 10b, and 10c show the construction of intermediate vectors containing autoantigen inserts into an HA secondary carrier which lacks membrane anchor codons.

-12-

Figures 11 and 12 show the construction of vectors designed to effect autoantigen secretion using the signal sequences of BAP and renin, respectively.

Figure 13 shows the construction of pGS20 and a diagram of its use in effecting integration of a desired sequence into the vaccinia virus TK gene.

Figure 14 shows the results of a gel analyzing the proteins produced by cells tranfected with expression vectors for the autoantigenic CTPs of the invention.

Figure 15a shows a comparison of human, rat, bovine, and dog B LH amino acid sequences. Figure 15b shows the location of peptides useful in species-specific vaccines, and the sequences of oligonucleotides encoding them.

Modes of Carrying Out the Invention

The invention, in one aspect, relates to methods and constructions which utilize the vaccinia system to introduce and express genes for autoantigens in vertebrate hosts. Heretofore, vaccinia has been used as a vehicle for DNA sequences which express proteins that are already immunogenic in the target host, and have not been employed to obtain expression of autoantigens that are altered to render them immunogenic. A particularly suitable "alteration" includes use of multimeric genes to produce multimeric antigens, so that the joined regions form the foreign epitopes. Immunogenicity is also provided when monomeric as well as multimeric genes inserted into DNA regions encoding known antigenic sites, such as those which reside in influenza hemagglutinin. The autoantigen will then be presented in a part of the protein known to be accessible to antibodies, and the

-13-

other hemagglutinin antigenic sites as well as the joining regions to the antigen region sequence represent the foreign epitopes. These and other constructions employing monomeric and multimeric genes in foreign proteins use these foreign proteins to carry and dispose the resulting chimeras to the infected cell surface or to secrete them from the infected cell. This transport makes the autoantigen-bearing chimeras accessible to the immune system.

As the proteins which result from in situ expression of these recombinant constructs are immunogens, the immunogens could also be produced outside the subject using recombinant techniques and administered as conventional protein vaccines. This recombinant expression includes the use of conventional procaryotic and eucaryotic expression systems and hosts, as well as transient expression from a recombinant intermediate shuttle vector using standard transformation into vaccinia-susceptible cells and infection with vaccinia virus.

As the vaccines are effective in producing antibodies which are immunospecific for the hormones involved in reproduction or for other indigenous proteins, these antibodies can, of course, be purified and used in immunoassays for the determination of the antigen.

A. Conferring Immunogenicity on Indigenous Proteins

At the heart of the invention is the use of recombinant techniques to create forms of indigenous proteins which are immunogenic, and which therefore behave as autoantigens.

These "autoantigens" are portions of or complete sequences of proteins normally made in the

-14-

host, and which proteins are thus immunologically privileged, unless they are made immunogenic. This immunogenicity can be obtained by linking the gene for an autoantigen which represents all or part of the immunologically privileged protein to a gene for an additional sequence, either a duplicate of the autoantigen or another secondary carrier protein, or both, so that the junction region between the autoantigen and its adjacent amino acids is clearly foreign to the host organism and is capable of conferring immunogenicity on the entire protein. The chimeric proteins produced by the inserts into vaccinia virus exhibit this property when produced in situ, or the same coding sequences can be produced using recombinant expression techniques in vitro and then used as conventional vaccines. In some cases, especially where the autoantigen is only a portion of an immunologically privileged protein, the autoantigen alone may be immunogenic.

20 In summary, the invention concerns recombiantly produced immunogenic forms of indigenous proteins. The immunogenic form obtained by constructing the gene encoding and producing either: a multimer which consists essentially of at least 2 repeating units of at least one epitope of the indigenous protein, or at least one epitope of the indigenous protein conjugated to a non-bacterial polypeptide, or a multimer which consists essentially of at least 2 repeating units of at least one epitope of the indigenous protein conjugated to an additional polypeptide sequence.

According to one embodiment of the invention, multimers of the desired autoantigen are created at the DNA level. The junction regions of the multimeric proteins constitute the foreign sequences which confer

-15-

immunogenicity on the resulting polymeric protein. This approach has the advantage of generating a relatively large immunogen with few irrelevant epitopes, and with a high concentration of the desired determinant, as compared, for example, with the more traditional conjugation of the desired hapten with a carrier protein. Alternative ways to create epitope polymers using polymerization agents which directly act upon the peptide, such as glutaraldehyde, result in denatured forms of the epitopes, and are therefore sometimes of marginal efficiency in producing the desired constructs. Using the techniques of the invention, the protein is "polymerized" at the DNA level, resulting in a recombinant polymer which more closely resembles the native peptide form. Recombinant multimers have been constructed from proinsulin (Shen, S., Proc Natl Acad Sci (USA) (1984) 81:4627-4631), and the multimers expressed in E. coli. These multimers were used to stabilize the recombinant protein, and were subsequently depolymerized to regenerate the monomeric form, which was the desired product. Recombinant multimers of the "CS" protein of the human malaria parasite have also been constructed (Young, J.F., et al, Science (1985) 228:958-962). These are peptides encoded by monomers, dimers and trimers of a XhoI fragment which therefore contain 16, 32, or 48 tandem copies of a repeated tetrapeptide sequence. The native protein contains 37 repeats of this tetrapeptide interspersed with 4 identical (to each other) tetrapeptides. The peptides are found to be immunogenic in mice.

The immunogenicity of the protein may also be enhanced by inserting it into the antigenic regions of a protein derived from an infectious material such as a

-16-

viral surface protein, or to a carrier which functions simply to increase the size of the peptide.

The "carrier" is a peptide sequence resulting in the production of a fusion protein between at least
5 one epitope of the desired autoantigen and a non-bacterially derived sequence capable of conferring immunogenicity on the epitope which provides the hapten for the immunogen. Again, the junction region represents unfamiliar sequence to the recipient, and the
10 additional peptide confers sufficient size, if needed, to raise antibodies against this determinant. The peptide sequences of the influenza hemagglutinin protein are particularly useful in providing the needed additional amino acid sequence.

15 A diagram of a typical hemagglutinin (HA) gene is shown in Figure 7. The particular map shown is of the antigenic variant A/Japan/305/57 (H2) as disclosed in Gething, M.J., et al, Nature (1980) 287:301. As
20 indicated in the figure, the gene encodes a signal sequence, a peptide containing approximately 550 amino acids followed by a membrane anchor sequence. The gene is framed by BalI, NdeI, and PstI restriction sites and contains several convenient restriction sites in the body of the amino acid sequence. The epitopes, either
25 as monomers or as multimers, may be ligated into the antigenic regions shown as A and B by providing blunt end restriction sites between codons in these regions using site specific mutagenesis. In addition, the naturally occurring restriction sites may be used to
30 result in proteins extended by the remaining portions of the HA carrier.

If the fused gene is expressed in culture outside the host to provide the fused protein, the HA sequences serve merely to provide the desired

-17-

immunogenicity on the construct. Of course, retaining the signal sequence and deleting the membrane anchor in the cultured cells will result in the ability to recover the desired protein from the culture medium. If

5 expressed in situ in the vaccinated host, the HA sequences serve functions more directly related to the presentation of the antigenic determinant to the host.

Of course, multimer encoding sequences may be ligated to DNA encoding carrier in a combination of the
10 foregoing methods.

An additional advantage may be obtained by the use of vaccinia as carrier for any autoantigenic gene constructed as described above in that the levels of immunogenicity may be adjusted through the nature of the
15 protein encoded. As stated above, if the autoantigen is inserted directly into vaccinia, the encoded autoantigenic protein will be produced as an intracellular protein with limited exposure to the immune system. Insertion into an antigenic region of a
20 viral protein will result in display of the antigen on the cell surface; similar enhancement may be obtained by using additional secondary carrier sequences which result in secretion from the infected cell or disposition on the cell surface.

25 The autoantigens are constructs of peptides which are involved in metabolic control of the subject host, especially hormonal control, and, in particular, fertility control.

The invention is illustrated below with respect
30 to specific useful embodiments. In one of these, coding sequences derived from β -hCG are made immunogenic and are thus useful in creating vaccines which prevent pregnancy in humans. In two other specific illustrations, GnRH and LH of known sequence are made in

-18-

a form to confer infertility on a variety of animals. Human GnRH is illustrated for use in animals in general, including dogs and cats. However, also preferred are other GnRH peptides of known sequence, such as the GnRH peptides shown for salmon and chicken in Figure 2. It should be noted that porcine and bovine sequences are identical with each other and with the human sequence. Presumably, in view of the high degree of sequence homology with species as distant as salmon, the GnRH native to dogs or cats or to other farm animals will at least have immunological cross-reactivity with the human and or chicken forms.

Both bovine, dog, and human LH β peptides are illustrated. These may have considerable cross-species reactivity, especially if regions of homogeneity are included. However, as illustrated below, species specific vaccines may also be constructed by recombinantly generating peptides or multimers of peptides which represent non-homologous regions.

In addition, other autoantigens which are fertility controlling autoantigens can also be used and include, for example, those derived from ovum proteins such as zona pellucida peptide; sperm antigens such as hyaluronidase and acrosin; the sperm-specific isozyme of lactose dehydrogenase, and the additional pituitary hormone follicle stimulating hormone (FSH).

In particular, certain approaches to fertility control are considered particularly desirable. For humans, vaccines which succeed in immunizing the subject based on the CTP portion of β HCG are particularly preferred, but it is also effective to utilize the entire β HCG subunit or other portion thereof, or the ovine β LH subunit or portion thereof to prevent pregnancy in humans. Thau, R.B., et al, Fertil Steril (1979) 31:200-204; ibid (1980) 33:317-320; ibid, Endocrinol

-19-

(1983) 112:277-283; and Yamamoto, Y., et al, J Reprod Fertil (1982) 4:295-311; J Reprod Immunol (1983) 5:195-202 have shown that ovine β LH is effective in preventing pregnancies in Rhesus monkeys. In addition, vaccines which confer immunity against FSH (Mougdal, N.R., Arch Androl (1981) 7:117-125; Mougdal, N.R., et al, Am J Reprod Immunol (1985) 8:120-124) and GnRH are administerable to decrease fertility in human males, although the administration of vaccines which raise antibodies against GnRH must be accompanied by administration of testosterone or other steroids which prevent loss of libido.

In animals, vaccines which are specific in raising antibodies against GnRH are preferred. Also particularly useful are vaccines which are species specific -- i.e., which are capable of preventing pregnancy or inducing sterility in only the species to which they are administered. These vaccines utilize epitopes which reside in regions of the hormones controlling reproduction which are non-homologous between species, particularly between humans and species to which humans must administer the vaccines. This may be especially desirable in constructing a vaccine useful in animals, as there would then be little danger of affecting the humans responsible for its administration. The illustration below sets forth an exemplary procedure whereby a region of low homology is chosen as the antigenic determinant for a vaccine.

In a converse application, the gene encoding inhibin, a hormone which affects fertility by lowering the ability of the pituitary to secrete FSH can be used; here the antibodies would increase the fertility of the host. See Olson, P.W., et al, Symposium on Endocrinology, Vet Clin NA: Small Animal Practice

-20-

(1984) 14:927-926. It also may be desirable to immunize the host against these and other hormones in order to regulate overproduction of these hormones and the resulting metabolic aberrations. In general, the autoantigens with which the invention is most concerned are "fertility controlling"--i.e., hormones which are involved in the physiological control of sex characteristics including behavior and fecundity.

In another context, it has been disclosed by Reddy, V.B., et al, Proc Natl Acad Sci USA (1985) 82:3644-3648, PCT Applications Publication Nos WO85/01958 and WO85/01959, that simultaneous enhanced expression of genes encoding both α and β subunits of the fertility hormones is achievable using an autonomously replicating vector in monkey cells. No production of the β chain of hCG was, however, observed if sequences encoding the α chain were omitted from the vector.

There are a large number of additional autoantigens to which it is desirable to generate neutralizing antibodies. A partial list of such autoantigens would include those corresponding to immunoglobulins generated by the victims of autoimmune diseases which are specific to the host's own tissues (where the neutralizing antibodies would suppress the autoimmunity) and those corresponding to the T24 bladder carcinoma growth factor (Taparowsky, E., et al, Nature (1982) 300:762), a protein generated by a human oncogene which causes the transformation of bladder cells (where the antibodies would retard the development of malignancy).

-21-

B. CTP Immunogens

The most preferred embodiment of vaccines against human pregnancy are those wherein the autoantigenic peptides are derived from the carboxy terminal peptide (CTP) of the β chain of human chorionic gonadotropin (β -hCG). Use of CTP-related peptides has several advantages:

First, while the CTP is derived from a human protein which is associated with a human hormone similar to certain others specified below, the CTP region is unique to hCG, thus minimizing the likelihood of unwanted interference with other essential hormones by antibodies raised against CTP. hCG itself is a glycoprotein of molecular weight 38 kd, of which 30% represents sugar. The protein has two dissimilar subunits, α and β -hCG, which are coupled by noncovalent linkage. The α subunits of hCG and of three other hormones--luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH) are identical in humans. hCG is secreted by the placenta; LH, FSH, and TSH are secreted by the pituitary. The β subunits of all four of these hormones show differences between them, but do retain some amino acid sequence homologies. The homologies are greatest between hCG and hLH β subunits; however, hCG contains an additional thirty amino acids extended beyond the 115 amino-acid residues in the β subunit of hLH. In general, positions 111-145 of β -hCG show no counterpart in hLH. Indeed, the peptide represented by positions 109-145 of hCG, conjugated to a carrier protein such as bovine gamma globulin or diphtheria toxoid, has been tested as an antifertility agent in baboons (Stevens, Serono Symposium (supra)).

-22-

A second advantage of utilizing the CTP region of B-hCG is that the hormone which it represents, as a placenta-derived hormone, is not generated directly by the host. Thus, antibodies raised against hCG which are not cross-reactive with FSH, LH, or TSH do not interfere with a hormone which might regulate the metabolism of the immunized subject. The role of hCG is presumed to be stimulation of steroid (progesterone) production by the corpus luteum following conception, until steroid production by the placenta itself can be established. Production of hCG by the placenta apparently begins within six days of conception, and is essential for the maintenance of pregnancy. If sufficient levels of hCG are not synthesized by eleven days, the corpus luteum regresses and normal menstruation occurs; the effect of this failure would presumably be mimicked by neutralizing antibodies. Immunization against hCG thus represents immunization against a non-maternal (although immunologically privileged) hormone which is apparently designed specifically for the maintenance of pregnancy and is presumptively not secreted for any other additional purpose.

A third advantage of using a partial sequence of hCG is that a vaccine using CTP as the immunogen provides sterility that is apparently reversible, as judged by preliminary tests on baboons. (Stevens, supra). This reversibility is thought to be due to the direction of T-cell memory not against native hCG but against the hCG/carrier protein joining regions. Therefore, production of the native hormone in intermittent pregnancies (which are presumably then terminated by the antibodies) is not likely to boost the anti-hCG response, and this has been shown in baboons (Stevens, supra). The ability to construct the CTP

-23-

portion as an unfamiliar protein may indeed account, in the previously disclosed constructions, for the immugenicity of what might otherwise be considered an indigenous protein.

5 (Indeed, this feature is not limited to CTP but is true of autoantigens in general. The subjects are not boosted by their own hormone or endogenous peptide production.)

While preliminary experiments have been
10 performed by Stevens (supra) indicating that the CTP region has possibilities as an antifertility vaccine, it has been apparent that a satisfactory formulation for administration of the immunogenic sequence has not been found. Vaccinia offers such a possibility by providing
15 a means for producing large quantities of immunogen in situ, as well as constituting a vehicle which has the benefit of collective experience through smallpox vaccinations and, more recently, through experimental immunizations against other diseases, as outlined
20 above.

This is not to say that alternate constructions are excluded. Fusion proteins containing the CTP monomer or multimer may be made using conventional recombinant techniques, and used in a manner common to
25 traditional subunit vaccines.

C. GnRH_n Immunogens

Gonadotropin releasing hormone (GnRH) is a decapeptide secreted by the hypothalamus and controls
30 the release of both luteinizing hormone (LH) and follicle stimulating hormone (FSH) in vertebrates (Fink, G., British Medical Bulletin (1979) 35:155-160). These hormones, in turn, control the levels of steroid hormones such as estradiol and progesterone. The amino

-24-

acid sequence of the decapeptide GnRH has been determined for several species, as shown in Figure 2, and the cDNA for human GnHRH has been cloned, so that its sequence is established beyond doubt (Seeburg, P.H.,
5 et al, Nature (1984) 311:666-668). (GnRH was formerly designated LHRH and is so named in the Seeburg paper.) As the peptide sequences for the GnRH from salmon and two such factors from chicken are known, DNA constructs encoding them are also easily prepared.

10 Both LH and FSH are significant in regulating the estrus cycles of dogs and cats, although different patterns are obtained. In the dog, luteinizing hormone peaks sharply at the onset of estrus and falls off as the period of estrus proceeds and is dissipated. FSH
15 also reaches a peak at this time, although the peak is slightly retarded from that of LH and the patterns are not precisely the same. The levels of estradiol (controlled by FSH) and progesterone (controlled by LH) also cycle. Therefore, the estrus cycle is destroyed by
20 preventing secretion of LH and FSH from the pituitary. This can be accomplished by suppression of GnRH activity through immunoreaction.

In cats, the orchestration is different, but the players are the same. The surge of LH essential for
25 ovulation is not released except upon sexual stimulation. However, the levels of LH and FSH that control sexual behavior and receptivity do cycle and are controlled by GnRH as in other vertebrates (Johnson, L.M. et al., Endocrinol (1981) 109:240-246). Thus,
30 prevention of LH and FSH secretion by suppressing the activity of GnRH will result in temporary sterility.

In addition, earlier studies have shown that antibodies to GnRH suppress sexual function in males (Fraser, et al, and Schanbacher, et al, supra). Thus,

-25-

vaccines relying on generation of GnRH as antigen are effective in male subjects as well.

An advantage of employing GnRH is the cross species effectiveness of a single vaccine. The amino acid sequence of GnRH is highly conserved among species. As shown in Figure 2, human, sheep, rat, pig, and cow GnRH are completely homologous; the most highly differing peptide is one of two derived from chickens which shows only three amino acid residue differences. All of these have biological activity in rats.

The constructs used in the present invention are derived from the human sequence but are modified by substituting the glutamine residue at the N-terminal position in place of the pyroglutamic acid. This substitution has the effect of retaining the glutamic acid structure but preserving the uncharged structure of the pyroglutamate. The resulting peptide thus does not require cyclization of the glutamic acid residue, and can be conveniently produced in the cytoplasm of the infected cell without concern for the presence of conditions to effect this processing, such as are ordinarily found in serum. The multimer as obtained from these constructs will have a C-terminal glutamine which mimics the amidation of the naturally occurring decapeptide, also obviating the need for subsequent processing.

With respect to obtaining suitable vaccines using GnRH as a base, the considerations discussed above with respect to CTP apply. Administration may be as a vaccinia virus construct or as a conventional subunit vaccine.

-26-

D. Luteinizing Hormone and Species Specificity

Vaccines are also illustrated below which are derived from human, bovine, and dog LH β chain sequences. As outlined above, LH is involved in maintenance of the corpus luteum, and although regulation of its level varies from species to species, it is essential for fertility in all. Therefore, antibodies capable of neutralizing LH are effective antifertility agents.

Figure 15a shows a comparison of the amino acid sequences of human, rat, bovine, and dog LH. Many regions are highly homologous, but the boxes designate regions where the differences from human sequence occur. Vaccines based on raising antibodies to LH can thus be made species-specific (or at least, ineffective in humans) by selecting these regions of low homology to obtain the autoantigen. In general, species specificity is obtained by selecting non-homologous regions, or vaccines can be made effective across species lines by using homologous regions. Figure 15b suggests peptides of interest for cow-specific and dog-specific vaccines.

The non-homologous peptides indicated are sufficiently short that genes encoding them can easily be made using the currently available chemical synthesis methods. Thus, the monomeric and multimeric units analogous to those of CTP and GnRH described above can be synthesized and manipulated in similar ways, either individually, or as cocktails which include two or three of the desired peptides. However, DNA capable of encoding major portions of either the bovine or human sequences can most conveniently be obtained by recovering a cDNA clone from libraries constructed using bovine or human pituitary mRNA. Complete genomic coding sequences for bovine LH (Virgin, J.B., et al, J Biol

Chem (1985) 260:7072-7077) and for human LH (Talmadge, K., et al. Nature (1984) 307:37-40) are known, and because of high sequence homology, the β -hCG cDNA released as a HindIII fragment from the pBR322 cloning vector described in Fiddes, J.C., et al. Nature (1980) 286:684-687 can be used as probe. The nucleotide sequence encoding the β chain of rat LH has also been disclosed (Chin, W.W., et al. Proc Natl Acad Sci (USA) (1983) 80:4649-4653). The cDNA sequences encoding cat or dog β LH can be retrieved from λ gt10 libraries prepared from pituitary mRNAs of the respective species.

Significant portions of the bovine, dog, and human LH sequences can be excised for the construction of autoantigenic forms as described in more detail in the illustration set forth below. For the bovine cDNA, naturally occurring HaeIII sites at amino acids 22 and 85 provide a convenient fragment; for the human cDNA, a MaeIII site at codons 111-112 can be employed along with an artificially introduced MaeIII site at codons 1-2. In all cases, these constructions are merely for the purpose of example, and constructions can be made using any major portion of the peptides. The manipulations to effect autoantigenicity - multimerization of the DNA, ligation into a carrier protein, production of a fusion protein, and so forth; and the means of administration - as a vaccinia borne expression system or as a protein or mixture of proteins produced by recombinant means, are similar to those described in connection with GnRH and CTP.

Antibodies raised against the immunogenic proteins of the invention, whether these proteins are produced for vaccine formulation or in situ, are useful in diagnosis and, if correctly prepared, in passive treatment. Conventional processes known in the art are

-28-

used to prepare these antibodies. Both the antibodies themselves and their immunospecific fragments, such as Fab fragments are thus useful.

5 E. Definitions

As used herein, "carboxy terminal peptide" or "CTP" refers to a region of β -hCG which has little in common with the three similar hormones FSH, TSH, and LH. β -hCG is a 145 amino acid peptide of known sequence. (Fiddes, J.C., et al, Nature (1980) 286:684-687). The approximately 30 amino acids representing positions 109-145 represents a unique determinant among this group. As used herein, CTP refers to a peptide having an amino acid sequence substantially similar to that represented by amino acids 109-145 of the native β -hCG. However, it is understood that minor modifications of the amino acid sequence may be made without affecting the immunological characteristics of the resulting peptide. Amino acid additions, deletions, and alterations can be made so long as they do not negatively affect the ability of the resulting peptide to elicit neutralizing antibodies against hCG itself. Such modifications include, but are not limited to, deletion of the cysteine residue at position 110 or replacement of the cysteine residue by serine or another amino acid which does not impair the immunogenicity of the resultant modified form, such as, for example, alanine, glycine, or valine. Deletion of this cysteine residue provides an improved form of the protein as the fortuitous formation of unwanted disulfides is thus prevented.

Also used in the vaccines of the invention are "multimers" of CTP which comprise repeated sequences of the particular CTP peptide employed. These multimers

-29-

may consist of multiples of 2-10 of the CTP peptide and are head-to-tail ligations of the monomer form. Thus the designation CTP_n, where n is 2-10, designates embodiments which contain 2-10 of the approximately
5 30-mers corresponding to CTP.

As used herein, "gonadotropin releasing factor" or "hormone" or "GnRH" refers to the hypothalamus product which is generally believed to effect the release of FSH and LH from the pituitary, as well as to
10 perform other regulatory functions relating to sexual activity, and which is typified by the amino acid sequences determined for various vertebrate species as shown in Figure 2. Where the context clearly so requires, GnRH also refers to the appropriate DNA
15 sequence which encodes the referenced peptide. As used herein, GnRH refers to a peptide having an amino acid sequence substantially similar to that represented in Figure 2; however, it is understood that minor modifications of the amino acid sequence may be made
20 without affecting the immunological characteristics of the resulting peptide, and the definition includes these resulting peptides. In the context of the present invention, amino acid additions, deletions, and alterations can be made so long as they do not
25 negatively affect the ability of the resulting peptide to elicit neutralizing antibodies against GnRH itself. Such modifications include, but are not limited to, replacing the pyroglutamic acid residue at position 1 of the human sequence by glutamine, and addition of a
30 glutamine residue at the C-terminus of the monomer or multimer. Such alterations do not impair the immunogenicity of the resultant modified form, and are specifically included within the definition. They, in fact, represent a preferred embodiment. It is also

-30-

clear from Figure 2 that the amino acid residues in positions 7 and 8 are the least conserved. Therefore altered forms of GnRH with substitutions in these positions are effective.

5 Also used in the vaccines of the invention are "multimers" of GnRH which comprise repeated sequences of the particular GnRH peptide employed. These multimers may consist of multiples of 2-10 of the GnRH peptide and are head-to-tail ligations of the monomer form. Thus
10 GnRH_n, where n is 2-10, designates embodiments which contain 2-10 of the decamer corresponding to GnRH. The multimers may contain an additional C-terminal amino acid, as is the case in the illustration below.

 As used herein, "human LH" or hLH, "bovine LH" or bLH, and "dog LH" or dLH refer to the respective
15 human and bovine hormones which are responsible for the maintenance of the corpus luteum. In general, the bLH, dLH, and hLH antigens used in the invention have the amino acid sequences shown in Figures 3a, 3b, and 4, or
20 significant portions thereof. Where the context clearly so requires, b-, d-, or hLH also refers to the appropriate DNA sequences which encode the referenced peptides. As used herein, bLH refers to a peptide having an amino acid sequence or portion substantially
25 similar to that represented in Figure 3a (dLH to that of Figure 3b; hLH to that of Figure 4); however, it is understood that minor modifications of the amino acid sequence may be made without affecting the immunological characteristics of the resulting peptide, and the
30 definition includes these resulting peptides. In the context of the present invention, amino acid additions, deletions, and alterations can be made so long as they do not negatively affect the ability of the resulting

-31-

peptide to elicit neutralizing antibodies against the appropriate LH itself.

Also used in the vaccines of the invention are "multimers" of the bovine, dog, and human LH which
5 comprise repeated sequences of the particular LH peptide employed. These multimers may consist of multiples of a significant portion of the appropriate LH sequence, such as, for example, the amino acids represented by residues 22-85 of the bovine LH 43-115 of dog LH, and 2-111 of
10 the human LH, and are head-to-tail ligations of the monomer form. Multiples of 2-10 monomers are used. Thus bLH_n , where n is 2-10, designates embodiments which contain 2-10 of the peptide representing residues 22-85 of the bLH sequence, or its functional equivalent;
15 ~~dLH_n and hLH_n designate the corresponding multimers~~ of the sequence of amino acids 43-115 of dog LH and 2-111 of hLH, respectively or their functional equivalents.

Generically, "Hormone_n" refers to a monomer
20 (if n is 1) or multimer of the appropriate portion of any particular hormone.

"Autoantigen" refers to a protein or portion thereof which is normally present in the targeted host, at least intermittently, and which normally does not
25 elicit an immune response from the host. Such autoantigens may ordinarily be made immunogenic only by using them as a hapten through conjugation to a suitable carrier. The carrier may simply be an extension of the amino acid chain; indeed, the extension may simply be
30 reproduction of the autoantigen in multiple units. The immunogenicity presumably results from the unfamiliar junction regions in this case. Similarly, immunogenicity results from the junction regions when the autoantigen is linked to sequences which serve a

-32-

secondary function, such as providing antigenic epitopes themselves, or serving carrier functions. On occasion, a portion of an immunologically privileged protein may, itself be immunogenic. The term "autoantigen" is used both to refer to the unaltered protein desired to be made immunogenic and to the autoantigen of interest in the immunogenic construct.

"Non-bacterial carrier" refers to a carrier protein which confers immunogenicity on an epitope of the indigenous protein by virtue of enhancing the size of the peptide and/or providing junction regions which contain amino acid sequences foreign to the host. The additional sequence may be any protein of non-bacterial origin, may include viral coat or core proteins,

~~secreted yeast proteins, such as invertase, which have~~ no homologous counterparts in mammals, and membrane bound and secreted proteins from protozoa, such as malaria. Particularly useful carrier proteins are those derived from influenza hemagglutinin. As used herein, "derived from" means that the amino acid sequence is the same as that for a portion containing at least 10 amino acids of the sequence of the source protein, and, of course, is not meant to mean physically obtained from that protein per se. Useful sequences derived from the hemagglutinin protein include portions of the antigenic regions and of the membrane anchor region of this protein.

"Secondary carrier" refers to a protein which is capable of transporting the autoantigen such as CTP or LH or its multimer to the surface of a cell infected with vaccinia virus containing the DNA encoding both the secondary carrier and the CTP or LH or its multimer, or of effecting the secretion of CTP or LH or its multimer from the infected cell. In this regard, the vaccinia

-33-

virus per se is generally referred to herein as a "carrier" for foreign sequences included in its genome. Thus foreign DNAs additional to the DNA encoding autoantigen, such as CTP or LH or its multimer, may
5 encode proteins which are secondary carriers for the immunogen. As described below, the influenza hemagglutinin protein is representative of secondary carriers useful in this regard. However, other proteins which are known to be transported to the surface of
10 infected cells may also be used. Such proteins include the neuraminidase peptide of influenza, rabies glycoprotein, and herpes simplex virus (HSV) glycoprotein D. Signal sequences operable in infected cells may be derived from a variety of sources including
15 preprorenin, preproinsulin, preprorennin, and bacterial alkaline phosphatase. An alternate method of secreting antigen utilizes the pre-S gene of hepatitis. The gene encoding the desired antigen is cloned into the pre-S gene, resulting in secretion of the chimeric protein,
20 which naturally aggregates to form an empty hepatitis virus coat. (Valenzuela, et al, Vaccines 85: Molecular and Chemical Basis of Resistance to Parasitic, Bacterial, and Viral Diseases, New York, Cold Spring Harbor Press, 1985, pp. 285-290).
25 "Operably linked" refers to juxtaposition so that the functionality of the elements is preserved. Thus, for example, promoters operably linked to coding sequences permit their expressions in suitable hosts. Signal sequences operably linked to other peptides (or,
30 at the DNA level, signal sequence encoding DNA operably linked to peptide encoding DNAs) permit the secretion of the subject peptide from the host cell.

It should be understood that "derived from", when referring to a DNA sequence or amino acid sequence,

-34-

indicates a correspondence in composition to the referenced material, and not necessarily actual physical derivation therefrom. For example, a DNA encoding a signal sequence which is derived from influenza hemagglutinin refers to a DNA constructed so as to encode a peptide sequence substantially similar to that which is found in influenza HA, and that sequence may be constructed by, for example, obtaining cDNA from the HA protein, by synthetic methods using automated oligonucleotide synthesis, or in any other manner designed to recreate a coding sequence for the desired peptide.

F. Preparation of Recombinant Vaccinia

The parameters for effecting the integration of foreign DNA into vaccinia are known in the art and are currently in a position for practical utilization. Briefly, the desired immunogen is transferred into a nonessential portion of the vaccinia genome by coinfecting host cells with both native vaccinia and a carrier plasmid which contains the foreign gene sandwiched between sequences homologous with the selected nonessential portion of the vaccinia genome. In addition, the foreign gene is provided with a vaccinia promoter, which will permit its expression under the influence of the vaccinia transcription and translation systems. A general purpose vector capable of housing the foreign gene has been disclosed by Moss, B., et al (Proc Natl Acad Sci (USA) (1983) 80:7155-7159). A diagram of this vector, pGS20, and its manner of use to obtain a recombinant vaccinia virus containing a desired foreign gene is shown in Figure 13.

pGS20 has a vector fragment derived from pBR328, a vector compatible with E. coli which contains

-35-

the E. coli origin of replication and the ampicillin resistance gene. The vector contains the promoter from the 7.5 k gene of vaccinia, excised as a 275 bp HincII/RsaI fragment (Venkatsen, et al, Cell (1981) 5 25:805-813), which is translocated into the EcoRI site of the vaccinia thymidine kinase (TK) gene. The TK gene is obtained as a HindIII/BglII fragment from the HindIII J fragment of vaccinia. Other restriction site modifications have been made for convenience, as shown 10 in Figure 13, and there are BamHI and SmaI restriction sites immediately downstream of the promoter to permit foreign gene cloning.

Thus, pGS20 is constructed as follows: pBR328 is digested with EcoRI, blunted, and religated. The 15 ~~resulting vector is then digested with HindIII and~~ ligated to the HindIII J fragment of vaccinia. The resulting plasmid is digested with BamHI and BglII and religated, thus eliminating the non-TK sequences, along with some unnecessary vector sequences from the insert, 20 as well as eliminating the BamHI and BglII sites. The vector is completed by inserting the HincII/RsaI 7.5 kD gene promoter fragment into the SmaI site of pUC8, excising the promoter as an EcoRI/BamHI fragment, and ligating it with the isolated EcoRI/SmaI/BamHI linker 25 fragment from pUC8 into EcoRI-digested vaccinia vector. Figure 13 also shows the nucleotide sequence in the region of the promoter/restriction site fragment junction for the CTP multimer embodiment.

In preparing the vectors of the invention, the 30 DNA encoding the desired immunogen is inserted into pGS20 using the restriction sites downstream of the promoter, as shown. The recombination vector is amplified in E. coli using transformation to Amp^R and then coinfectd along with wild-type vaccinia into CV-1

-36-

cells. Unlike other large DNA animal viruses, vaccinia transcribes and replicates its genome in the cytoplasm of infected cells. Many of the enzymes involved in its nucleic acid metabolism, such as DNA and RNA
5 polymerases, enzymes to cap, methylate, and polyadenylate RNA, as well as thymidine kinase, are encoded in its own genome. Indeed, protein-free vaccinia is noninfective--since it encodes its own transcriptase and apparently cannot use the
10 transcriptase used by its eukaryotic host, it cannot synthesize required proteins using its DNA alone in combination with the host cell machinery.

Use of the TK-encoding sequences to effect recombination is particularly desirable, as not only is
15 this a nonessential portion of the vaccinia genome, but a selectable marker is provided for cells containing the recombined vaccinia--i.e., when the foreign DNA is inserted, the TK gene is inactivated, and the tk-recombinant viruses can be selected by plaque assay on
20 tk- cells in the presence of 5-bromodeoxyuridine (BUdR). As shown in Figure 13, tk- recombinant virus plaques can be selected yielding the desired recombinant vaccinia. Additionally, tk- vaccinia have been shown to be 10^5 to 10^6 times less virulent in animals as
25 compared to wild-type vaccinia, indicating the potential for an increased safety factor in humans.

While pGS20 is a convenient illustrative vector, it is understood that alternative constructions involving other vaccinia promoters in other nonessential
30 regions of the gene may be used (Moss, et al, Gene Amplification Analysis, Vol. III, Pappas, T.K., et al, eds. (1982) New York, Elsevier, pp. 201-213; Mackett, M., et al, J Virol (1984) 586-864).

-37-

The recombined vaccinia can also be administered as a killed vaccine. Subject cells in tissue culture are infected with vaccinia in vitro, and then heat-killed. The attenuated, killed vaccinia are then administered with an adjuvant as are conventional vaccines.

G. Alternate Modes of Vaccine Construction and Administration

The autoantigenic constructions of the invention need not necessarily be administered as vaccinia borne DNA encoding them. They may also be produced as proteins using standard means of recombinant protein production and administered as conventional vaccines. The choice of route is dependent, of course, on the subject species and on the nature of the antigen.

Standard means for recombinant expression of DNA sequences are set forth below. In addition, the pGS20 vectors containing the desired autoantigen sequences may be used as expression vectors in vitro by using the transient expression which occurs when susceptible cells transfected with this vector are infected with vaccinia virus. The protein produced may be obtained from the cell lysate or from the supernatants, depending on whether construction of the vectors results or does not result in secretion.

If the autoantigens are administered as proteins, they are formulated into conventional dosage forms as injectables, either as solutions, suspensions, emulsions, or solids for reconstitution. Suitable excipients include water, saline, dextrose solution, glycerol solution, Hank's solution, Ringer's solution, and the like. Formulation techniques for protein containing vaccines are found in standard reference

-38-

works such as Remington's Pharmaceutical Sciences, Mack Publishing Co. Easton, PA, latest edition. Adjuvants may also be used, such as aluminum hydroxide, muramyl dipeptides, interferon, and immune-stimulating peptide regions. Vehicles can include squalene:aracel mixtures, croton oil, or other suitable compositions.

In those instances in which small peptides are used as autoantigens, such as, for instance, the peptides representing the non-homologous regions of LH, these are typically linked to carrier proteins, either at the protein or DNA level. Techniques for ligation at the DNA level are standard: mediation of the reaction using an appropriate ligase is used, as described below. At the protein level, a variety of chemistries can be used, including utilization of commercially available linkers such as SPDP and SMCC. The carrier protein in this case is an antigenically neutral peptide appropriate to the species, such as, for example, BSA for use in cattle, or HSA or tetanus toxoid for use in humans.

H. Production of Antibodies

As the vaccines of the invention are successful in raising antibodies which recognize epitopes associated with important regulators of metabolism, they provide a useful means to obtain components of immunoassays for such antigenic species. Also included in the invention, therefore, are antibody preparations obtained as a result of immunization with the vaccines provided. The antibody preparations may be obtained directly from the antisera as polyclonal preparations, or the peripheral blood cells or spleen cells from an immunized mammal may be used as fusion partners and monoclonal immunoglobulin producing hybridomas

-39-

obtained. For use in immunoassay, only the portion of the immunoglobulin related to specificity, and the invention therefore includes the Fab regions of the antibodies produced.

5 The antibody or Fab portion can be labeled or attached to solid support, or otherwise modified as is understood in the art in preparation for use in the assays.

10 Standard Methods

1. Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employs standard
15 ~~ligation and restriction techniques which are well~~
understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

Site specific DNA cleavage is performed by
20 treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g., New England Biolabs,
25 Product Catalog. In general, about 1 µg of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 µl of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate.
30 Incubation times of about one hour to two hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from

-40-

aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general
5 description of size separations is found in Methods in Enzymology (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of E. coli DNA polymerase I (Klenow) in the presence of the four
10 deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 min at 20 to 25°C in 50 mM Tris pH 7.6, 50 mM NaCl, 6 mM MgCl₂, 6 mM DTT and 1 mM dNTPs. The Klenow fragment fills in at 5' sticky ends but chews back protruding 3' single strands, even though
15 the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the sticky ends. After treatment with Klenow, the mixture is extracted with phenol/chloroform and
20 ethanol precipitated. Treatment under appropriate conditions with S1 nuclease or mung bean nuclease results in hydrolysis of any single-stranded portion.

Synthetic oligonucleotides are prepared by the method of Efimov, V. A., et al (Nucleic Acids Res (1982)
25 6875-6894), and can be prepared using commercially available automated oligonucleotide synthesizers. Kinasing of single strands prior to annealing or for labeling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 1
30 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol, 1-2 mM ATP, 1.7 pmoles γ 32P-ATP (2.9 mCi/mole), 0.1 mM spermidine, 0.1 mM EDTA.

-41-

Ligations are performed in 15-50 μ l volumes under the following standard conditions and temperatures: 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 μ g/ml BSA, 10 mM-50 mM NaCl, and either 40 μ M ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 μ g/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations (usually employing a 10-30 fold molar excess of linkers) are performed at 1 μ M total ends concentration.

In vector constructions employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIP) in order to remove the 5' phosphate and prevent religation of the vector. Digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na^+ and Mg^{+2} using about 1 unit of BAP or CIP per μ g of vector at 60° for about one hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

2. Site-Specific Mutagenesis

For portions of vectors derived from cDNA or genomic DNA which require sequence modifications, site specific primer directed mutagenesis is used. This is conducted using a primer synthetic oligonucleotide complementary to a single stranded phage DNA to be

-42-

mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the
5 resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells which harbor the phage.

10 Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. The resulting plaques are hybridized with kinased synthetic primer and then washed at a temperature which permits
15 hybrids of an exact match to remain, but at which the mismatches with the original strand are washed off. Plaques which remain hybridized to the probe at the stringent wash temperature are then picked, cultured, and the DNA recovered. Details of site specific
20 mutation procedures are described below in specific examples.

For probing, plaques are screened by replicating the plaques onto duplicate nitrocellulose filter papers (S & S type BA-85) and infected cells are
25 allowed to grow at 37°C for 14-16 hr on L agar containing 15 µg/ml tetracycline. The colonies are lysed with 10% SDS and the DNA is fixed to the filter by sequential treatment for 5 min with 500 mM NaOH/1.5 M NaCl, then 0.5 M Tris HCl(pH 8.0)/1.5 M NaCl followed by
30 2 x standard saline citrate (SSC). Filters are air dried and baked at 80°C for 2 hr.

For synthetic (15-30 mer) oligonucleotide probes, the duplicate filters are prehybridized at 42°C for 2-8 hr with 10 ml per filter of oligo-hybridization

-43-

buffer (6 x SSC, 0.1% SDS, 1 mM EDTA, 5x Denhardt's, 0.05% sodium pyrophosphate and 50 µg/ml denatured and sheared salmon sperm DNA).

5 The samples are hybridized with kinased oligonucleotide probes of 15-30 nucleotides under conditions which depend on the composition of the oligonucleotide. Typical conditions employ a temperature of 30-42°C for 24-36 hr with 5 ml/filter of this same oligo-hybridization buffer containing probe.

10 The filters are washed two times for 15 min at 23°C, each time with 6 x SSC, 0.1% SDS and 50 mM sodium phosphate buffer at pH 7, then are washed once for 2 min at the stringent wash temperature with 6 x SSC and 0.1% SDS. Typically, the stringent wash temperature for

15 oligonucleotides of 16-24 bases with from 1 to 3 mismatches will be 40-70°C, and can most easily be determined by successive washes of the hybridized filter. For example, the hybridized filters can be washed first at 40°C, then at 50°C, then at 60°C, and

20 then at 70°C, with air drying of the filter and autoradiography at -70°C overnight between each wash.

3. Verification of Construction

In the constructions set forth below, correct

25 ligations for plasmid construction are confirmed by first transforming E. coli strain MC1061 (Casadaban, M., et al, J Mol Biol (1980) 138:179-207) or other suitable host with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline

30 or other antibiotic resistance or using other markers depending on the mode of plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of Clewell, D. B., et al, Proc Natl Acad Sci (USA) (1969) 62:1159,

-44-

optionally following chloramphenicol amplification (Clewell, D. B., J Bacteriol (1972) 110:667). The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy method of Sanger, F., et al, Proc Natl Acad Sci (USA) (1977) 74:5463 as further described by Messing, et al, Nucleic Acids Res (1981) 9:309, or by the method of Maxam, et al, Methods in Enzymology (1980) 65:499.

10 4. Hosts Exemplified

Host strains used in cloning and expression herein are as follows:

For cloning and sequencing, and for expression of construction under control of most bacterial

15 promoters, E. coli strain MC1061 was used.

For M13 phage recombinants, E. coli strains susceptible to phage infection, such as E. coli strain JM101 are employed.

For eucaryotic expression, the monkey cell
20 line, CV-1 is used.

5. Recombinant Expression Systems

Both procaryotic and eucaryotic systems may be used to express the autoantigen encoding sequences;

25 procaryotic hosts are, of course, the most convenient for cloning procedures. Procaryotes most frequently are represented by various strains of E. coli; however, other microbial strains may also be used. Plasmid vectors which contain replication sites, selectable
30 markers and control sequences derived from a species compatible with the host are used; for example, E. coli is typically transformed using derivatives of pBR322, a plasmid derived from an E. coli species by Bolivar, et al, Gene (1977) 2:95. pBR322 contains genes for

-45-

ampicillin and tetracycline resistance, and thus provides multiple selectable markers which can be either retained or destroyed in constructing the desired vector. Commonly used procaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the β -lactamase (penicillinase) and lactose (lac) promoter systems (Chang, et al, Nature (1977) 198:1056) and the tryptophan (trp) promoter system (Goeddel, et al Nucleic Acids Res (1980) 8:4057) and the lambda derived P_L promoter and N-gene ribosome binding site (Shimatake, et al, Nature (1981) 292:128).

In addition to bacteria, eucaryotic microbes, such as yeast, may also be used as hosts. Laboratory strains of Saccharomyces cerevisiae, Baker's yeast, are most used although a number of other strains or species are commonly available. Vectors employing, for example, the 2 μ origin of replication of Broach, J. R., Meth Enz (1983) 101:307, or other yeast compatible origins of replication (see, for example, Stinchcomb, et al, Nature (1979) 282:39, Tschumper, G., et al, Gene (1980) 10:157 and Clarke, L, et al, Meth Enz (1983) 101:300) may be used. Control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes (Hess, et al, J Adv Enzyme Req (1968) 7:149; Holland, et al, Biochemistry (1978) 17:4900). Additional promoters known in the art include the promoter for 3-phosphoglycerate kinase (Hitzeman, et al, J Biol Chem (1980) 255:2073). Other promoters, which have the additional advantage of transcription controlled by growth conditions and/or genetic background are the promoter regions for alcohol dehydrogenase 2,

-46-

isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, the alpha factor system and enzymes responsible for maltose and galactose utilization. It is also believed terminator sequences are desirable at the 3' end of the coding sequences. Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes.

It is also, of course, possible to express genes encoding polypeptides in eucaryotic host cell cultures derived from multicellular organisms. See, for example, Axel, et al, 4,399,216. These systems have the additional advantage of the ability to splice out introns and thus can be used directly to express genomic fragments. Useful host cell lines include VERO and HeLa cells, and Chinese hamster ovary (CHO) cells.

Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, the commonly used early and late promoters from Simian Virus 40 (SV 40) (Fiers, et al, Nature (1978) 273:113), or other viral promoters such as those derived from polyoma, Adenovirus 2, bovine papilloma virus, or avian sarcoma viruses. The controllable promoter, hMTII (Karin, M., et al, Nature (1982) 299:797-802) may also be used. General aspects of mammalian cell host system transformations have been described by Axel (supra). It now appears, also that "enhancer" regions are important in optimizing expression; these are, generally, sequences found upstream or downstream of the promoter region in non-coding DNA regions. Origins of replication may be obtained, if needed, from viral sources. However, integration into the chromosome is a common mechanism for DNA replication in eucaryotes.

-47-

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, S.N., Proc Natl Acad Sci (USA) (1972) 69:2110, or the RbCl_2 method described in Maniatis, et al, Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor Press, p. 254 and Hanahan, D., J Mol Biol (1983) 166:557-580 may be used for procaryotes or other cells which contain substantial cell wall barriers. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology (1978) 52:546, optionally as modified by Wigler, M., et al, Cell (1979) 16:777-785 may be used. Transformations into yeast may be carried out according to the method of Beggs, J.D., Nature (1978) 275:104-109 or of Hinnen, A., et al, Proc Natl Acad Sci (USA) (1978) 75:1929.

6. Additional cDNAs

Additional constructs can be made taking advantage of homologies between the DNA sequences disclosed herein and the corresponding hormones in other species. Thus, RNA may be prepared from the corresponding gland, such as the pituitary for LH, in a previously uninvestigated species, for example, rabbit, and a cDNA library prepared and probed. Effective techniques for extraction for mRNA are known in the art and generally employ affinity chromatography with oligo dT to obtain polyadenylated messenger RNA. The RNA is then used to obtain a cDNA library, for example, by cloning into the phage vector $\lambda\text{gt}10$ (Hunyh, T.V., et al, in DNA Cloning Techniques, a Practical Approach (1984) D. Glover, ed) or into a phage vector library capable of expression in bacteria using $\lambda\text{gt}11$ as

-48-

described by Young, R.A., et al, Proc Natl Acad Sci USA
(1983) 80:1194. The resulting cDNA can then be probed
with denatured DNA sequences derived from the hormone
sequences disclosed herein, for example, bovine LH B
5 chain or the dog LH sequence.

Examples

The following examples are intended to
illustrate the invention but not to limit its scope.

10

Example 1

Construction of Autoantigen-Encoding Sequences

A. CTP Multimers

15 ----- The construction of the desired genes is shown
in Figure 1. The major portion of the gene is isolated
as the BstNI/Sau3A fragment encoding amino acids 117-141
(Fiddes, J.C., et al, Nature (1980) 286:684-687). The
5' end and 3' end of the gene are synthesized chemically
20 in order to provide desired overhang for polymerization,
and, if desired, codon alteration. For all of the CTP
fragments illustrated, the middle portion and C-terminal
portions are identical; differences, however, occur in
the upstream segment, depending on whether the native
25 sequence is used or that altered in the 110 position.

For each of the three constructs, the three
pertinent fragments are ligated to obtain the β -hCG CTP
monomer and the desired product is isolated using
polyacrylamide gels. Multimers are obtained by
30 self-polymerization and separation of the various
multimers on gels, e.g., the 3-mer (MW = 12 kD); 4-mer
(MW = 16 kD); 6-mer (MW = 24 kD), or larger multimers.

The desired CTP_n constructs are blunt-ended
using Klenow and the appropriate dNTPs and either

-49-

amplified in E. coli using intermediate vectors (Example 3) before direct recombination into vaccinia or are cloned into secondary carrier protein-encoding sequences before amplification in E. coli and subsequent recombination into vaccinia.

B. GnRH Multimers

The DNA sequences of the desired genes are shown in Figure 2. The double stranded portion encoding the monomer is obtained by annealing the two complementary strands, which strands are synthesized employing standard automated techniques. The monomer is isolated on a gel, and confirmed to be of the correct size. Multimers are obtained by self-polymerization and separation of the various multimers on gels, e.g., the 8-mer (243 base pairs); 10-mer (303 base pairs); 15-mer (453 base pairs), or larger multimers.

The desired GnRH_n constructs are blunt-ended using Klenow and the appropriate dNTPs and either amplified in E. coli using intermediate vectors (Example 3) before direct recombination into vaccinia or cloned into secondary carrier protein-encoding sequences before amplification in E. coli and subsequent recombination into vaccinia.

C. LH Multimers

The complete cDNA sequences encoding the β chains of both human luteinizing hormone (hLH) and of the bovine counterpart (bLH) are known (see Figures 3 and 4), and chemical synthesis could therefore be used to construct the desired oligonucleotides. However the constructions described below utilize portions of such length that although direct chemical synthesis is possible, it is more convenient to obtain the cDNA

-50-

encoding them from standard libraries in λ gt10 prepared from human and bovine pituitary mRNA. The libraries are prepared by standard methods using EcoRI linkers, and are probed using the β -hCG cDNA insert removed with HindIII from the pBR322 cloning vector disclosed by Fiddes, J, et al, Nature (supra). (The LH and hCG β peptides are highly homologous.) The cDNA inserts are then transferred from the lambda phage vectors to the EcoRI site of pBR322 to obtain the cloning vectors pH LH and pBLH for the human and bovine forms of the protein, respectively.

Similarly, cDNA encoding the β peptides of dog LH was obtained from a λ gt10 cDNA library prepared using dog pituitary mRNA as template. The library was probed with the same HindIII HCG β peptide described above, and a single clone containing the coding sequence from amino acid 18 to the stop codon after amino acid 121 was retrieved. This clone, in combination with another similarly retrieved from the library, permitted deduction of the entire amino acid sequence for dog β LH, as well as the 18 residue signal sequence preceding the N-terminal serine residue of the mature protein. The complete cDNA sequence encoding the mature protein and its signal peptide, along with the deduced amino acid sequence is shown in Figure 3b.

The single clone containing the sequence encoding amino acids 18-121 was retrieved under hybridization conditions which include 30% formamide at 42° followed by washing 2 times at 50°C in 2XSSC. The EcoRI insert in the λ gt10 clone was then moved into the EcoRI site of pBR322 to obtain the cloning vector pDLH for the dog protein.

In a manner similar to that described above for retrieval of dog LH, the cDNA encoding the cat protein

-51-

is prepared from cat pituitary mRNA and cloned into pBR322 to obtain pcLH.

The multimers are prepared directly in the vectors. For the bovine LH, a repeating sequence
5 encoding amino acids 22-85 is inserted into the full length sequence in pbLH by repeatedly inserting the 192 bp fragment obtained by HaeIII digestion of pbLH into the StuI site which coincides with the HaeIII site at the codon for amino acid 22 (see Figure 3). While the
10 blunt ended HaeIII fragment can be inserted in two possible orientations, the correct construction is distinguishable by restriction analysis, as it produces an EcoRI/StuI digestion fragment 192 bp shorter than the incorrect construction. The HaeIII fragment insertion
15 is repeated for the desired number of (n-1) times, resulting in the 22-85 region peptide repeated n times (the StuI digested vector provides the additional repeat). Preferred values for n are 3-10.

For the human multimers, the EcoRI insert of
20 phLH is first transferred to the EcoRI site of M13mp8 ~~for site specific mutagenesis to provide an MaeIII site~~ at amino acids 1-2 to complement that already present in the cDNA bridging the codons for amino acids 111-112. (MaeIII is obtained by the purification procedure of
25 Schmid, K., et al, Nucleic Acids Res (1985)). The primer is CCGTAGGTCACTGGGCGAAGCC as shown in Figure 4. The single stranded mutagenized phage is then hybridized to universal primer, extended with Klenow and the four dNTPs (30 minutes at room temperature), and
30 cleaved with MaeIII to obtain the desired fragment extending between the new MaeIII site at codons 1-2 of the mature sequence and the naturally occurring MaeIII site at 111-112. This fragment is ligated by self polymerization in a manner analogous to that described

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-52-

for CTP or GnRH above, frame and orientation are maintained in the polymerization. For consistency, it is considered that these products of polymerization contain n-1 repeats. The multimers are then inserted
5 into the MaeIII cleaved pHLH to obtain a cloning vector having n repeats (the extra repeat being supplied by the host vector). Preferred values of n are 3-10.

The dog multimer is constructed using the cDNA region between codon 43 (a RsaI site) and codon 115 (a
10 SmaI site). The multimer is prepared by repeatedly inserting the blunt RsaI/SmaI fragment into SmaI cleaved pdLH and its resultants. Insertion of the RsaI/SmaI fragment into the SmaI site of pdLH, when in the correct orientation, destroys the RsaI site of the insert and
15 recreates the SmaI site at the 3' end, thus permitting the repetitive insertion. The multimerized region can then be released by digestion with RsaI/SmaI as the only SmaI site remaining is at the 3' end of the multimerized region. The relevant portions of the junction regions
20 are shown in Figure 3b. As above, preferred values of n are 3-10.

Example 2

Preparation of Cloning Vectors

25 Figure 5 shows the construction of the cloning vector pVA1 derived from pKK223-3 (Brosius, J., et al, Proc Natl Acad Sci (USA) (1984) 81:6929-6933). Construction of pVA2, derived from pKT19 and pKT41 (Talmadge, K., et al, Gene (1980) 12:235-241), is also
30 shown in Figure 5.

Both constructions result in vectors which contain as an insert the synthetic nucleotide shown below:

-53-

NcoI
BamHI BalI NdeI SmaI
 TGGATCCATGGCCAGGCATATGATTGATTGACCCGGG
ACGTACCTAGGTACCGGTCCGTATACTAACTAACTGGGCCCTTAA
 5 No PstI ter ter ter EcoRI

This sequence, designated herein the NoPst/R oligomer, contains termination codons in all three possible reading frames, as well as convenient restriction NcoI, NdeI, and BalI sites for insertion of coding sequences. It also includes BamHI and SmaI sites at either end suitable for insertion into the pGS20 intermediate vector for vaccinia recombination.

In the constructions described, the NoPst/R oligomer is inserted into suitable host carrier vectors. The resulting cloning vectors ideally should lack NdeI and BalI sites to permit those contained in the NoPst/R oligomer to be used uniquely for subsequent insertion of the desired coding sequences; as BalI is a troublesome enzyme, the alternate upstream sites in the NoPst/R insert, the NcoI and BamHI sites, can be used in its place. The cloning vector also must carry markers, such as those conferring antibiotic resistance, as well as, preferably, a high copy number origin of replication. Two such vectors were constructed, pVA1, which fits the foregoing criteria but contains a HincII restriction site in the Amp^R gene, and pVA2, which contains an intact Tet^R gene and has no HincII or PvuII site, which sites are needed to be absent, as will be seen below, for certain manipulations of an inserted HA. Thus, initial cloning may be done in pVA1, but for constructions which require the use of HincII and PvuII sites, the HA gene must be moved into pVA2.

-54-

Construction of pVA1

Referring to Figure 5, pKK223-3 is first treated to remove unneeded DNA sequences and to remove an extraneous PvuII site. pKK223-3 is digested with EcoRI and PvuII, blunt-ended with Klenow, and then religated to generate pAL1, a 2.6 kb plasmid with the required origin of replication and the Amp^R gene. pAL1 is then treated with EcoRI and PstI and ligated to the NoPst/R oligomer to obtain pVA1.

Construction of pVA2

Referring to Figure 5, to prepare pVA2, pKT19 and pKT41 were digested with EcoRI and SphI, and the small pKT19 fragment isolated from a polyacrylamide gel was ligated to the large pKT41 fragment, also isolated from a 1% agarose gel, to obtain pKT1941. pKT1941 was digested with PvuII, ligated to a KpnI linker, cleaved with KpnI, and the religated to eliminate the PvuII site, creating pKT1941.1. The BalI and NdeI sites present in pKT1941.1 were eliminated in a two step procedure, the first step comprising digestion with NdeI, blunting with Klenow, ligating to KpnI linker, and religation of the plasmid; the second step analogously comprising digestion with BalI, ligating to KpnI linker, and religation to obtain pKT1941.3. This vector was digested with EcoRI and PstI and ligated with a tenfold molar excess of the synthetic fragment above to obtain pVA2.

Example 3Preparation of Intermediate Vectors for Intracellular Expression of Multimers

Intermediate vectors carrying the multimeric genes prepared above for intracellular expression when

-55-

recombined into vaccinia are prepared by inserting the desired multimeric genes into the pVA1 or pVA2 vectors of Example 2. pVA1 or pVA2 is cleaved with NcoI and NdeI and blunt-ended with Klenow and the four dNTPs; the
5 multimer-encoding sequences are correspondingly blunt-ended and ligated into the cleaved vector.

For the multimer of CTP, the synthetic polymerized multimers described in Example 1A are blunted and ligated into the NcoI(repair)/NdeI(repair)
10 digested vectors. The resulting intermediate vectors, pVA1/CTP_n and pVA2/CTP_n, where n is the number of monomeric genes, are used as a source for the CTP_n sequences in preparing recombinant vaccinia, as set forth below.

15 For the multimers of GnRH, self-polymerized, blunted GnRH DNA sequences of Example 1B are inserted analogously to obtain pVA1/GnRH_n and pVA2/GnRH_n.

For the multimers of the LH gene portions, suitable forms of the multimers can be inserted into the
20 NcoI(blunt)/NdeI(blunt) vectors to provide, for example, pVA1/hLH_n and pVA2/bLH_n. However, in both cases, a properly placed NcoI site is needed, as shown in Figures 3 and 4, to place the upstream portion in reading frame with the start codon provided by the repaired NcoI from
25 the vector. The multimer inserts are therefore removed from pBLH and pH LH multimer-containing vectors by EcoRI digestion and placed into the EcoRI site of M13mp8 for site specific mutagenesis, the replicative forms obtained, and the NcoI(single strand
30 removed)/EcoRI(blunt) fragments removed for insertion into pVA1 or pVA2.

The above vectors can be referred to generically as pVA/Hormone. The hormone monomers or multimers, preceded by an ATG start codon obtained by

-56-

repair of the NcoI site and terminated by the termination codons provided in the NoPst/R fragment are transferred to pGS20 as a BamHI/SmaI fragment. Transient recombinant expression may be obtained using
5 cells transfected with the pGS20 derivative and infected with vaccinia, or the vaccinia recombinants from these cells may be used directly as vaccines. In addition, the monomer or multimer hormone fragments may be ligated into vectors carrying the coding sequences for other
10 proteins, as described below.

Example 4

Preparation of Derivative Vectors Containing HA

Three HA-containing vectors were prepared.
15 pHA1 is used for insertion of the multimeric sequences into the mutagenized antigenic sites of the HA gene. As described below, it is provided with blunt end-resulting cleavage sites in the antigenic regions by site directed mutagenesis. In the constructions for GnRH and CTP,
20 which are described in detail, these sites are located between codons, so that the reading frame for these multimers, which also terminates at codon boundaries, is maintained. pHA2 permits insertion using native restriction sites to ligate these multimers to the
25 membrane anchor and signal sequences, the restriction sites also provide blunt-end termination at codon boundaries. pHA3 permits use of the EcoRI sites in the HA gene for ligation of the LH sequences, which have an extra bp beyond the codon boundary at either end.
30 The construction of pHA1 and pHA2 is shown in Figure 6. The HA gene from pSVL-HA8 or pSVE-HA3 (Gething, M.J., Nature (1981) 293:620-625) was excised using HindIII/BamHI digestion. The resulting fragment

-57-

contains the entire gene. A map of the HA gene is shown in Figure 7.

As shown in Figure 6, the HA gene fragment was ligated into HindIII/BglII digested pW6, a modified pBR322 which retains Amp^R, but which contains a linker providing the desired restriction sites in the region of the deleted Tet^R resistance gene. (To prepare pW6, pBR322 was digested with EcoRI and NruI and ligated with an EcoRI/blunt synthetic oligonucleotide, designated herein R/NoNru, and shown in the Figure, which contains BamHI, HindIII, BglII, and SmaI sites.) The pHA1 vector therefore contains the entire HA gene framed by BamHI and SmaI sites.

Because the remaining backbone fragment containing the Amp^R gene from pBR322 contains unwanted restriction sites, it can be replaced by BamHI/NdeI digested pVA2. The HA gene from pHA1 is excised as a BamHI/NdeI fragment, which contains all of the gene except the codon 611 and the stop codon; the stop codon in pHA2 is provided by the downstream portion of the NoPst/R oligomer of pVA2.

The pHA3 vector is prepared from pHA2 by digesting with EcoRI, blunting, and religating to eliminate the EcoRI site of the linker before insertion of the HA gene as above.

The pHA1 cloning vector containing the HA gene can be conveniently modified to receive the in-frame multimer fragments by creating the restriction sites shown below, except for the HpaI site, in the A and B antigenic regions. pHA2 can be modified to provide the HpaI site. This is accomplished through site-specific mutagenesis using the site-specific primers:

-58-

5'-GTGTCTGGTAGGCCTTCATTTTTC-3', which provides a StuI site between codons 139 and 140 in the A antigenic regions, as shown (HA/Stu);

5'-GCGCGGTGTCGCGAAATCCATCAT-3', which provides an NruI site between codons 137 and 138 of the A antigenic region (HA/Nru);

5'-TCTGGCTGGTTAACGAAGGATC-3', which provides a HpaI site between codons 150 and 151 in the B antigenic region; and

5'-TCCCATTGATATCACAGAACAAA-3', which creates an EcoRV site between codons 182 and 183 of the B antigenic region (HA/RV).

The mutagenesis is conducted by excising the BamHI/SmaI insert from pHA1 or pHA2, ligating the insert into BamHI/SmaI-digested M13mp8 for the mutagenesis, then religating the altered phage inserts into the original vectors.

The exemplified restriction sites above are designed to provide for in-frame insertion of the CTP and GnRH multimers prepared as described herein. Alternate, analogous sites are required for insertion of the LH sequences described which are excised as fragments in different reading frames.

25

Example 5

Preparation of HA/Multimer Constructs

pHA/Nru, pHA/Stu, pHA/Hpa, and pHA/RV are digested with NruI, StuI, HpaI, and EcoRV respectively (all provide blunt-ended cleavage fragments) and ligated with the both the monomer and multimer blunted gene fragments of CTP or GnRH prepared in Example 1. As shown in Figure 8, these inserts are in correct reading frame with the HA coding sequence, since the enzymes digest between codons, and the vectors thus contain

-59-

chimeric genes with the desired hormone derived epitopes encoded into the antigenic regions of HA. The vectors are designated, for example, pCTP_n/HA/Stu, pGnRH_n/HA/Nru, or phCTP_n/HA/Hpa or generically, pHormone/HA/site. (It will be recalled that these particular restriction sites are not suitable for the illustrated LH multimers.) As the HA proteins are carried to the surface of the infected cell, expression of the chimeras recombined into vaccinia results in exposure of these epitopes at cell surfaces.

Example 6

Preparation of Intermediate Vectors Carrying Chimeras Between HA Signal Sequence and Membrane Anchor

15 An alternative approach to providing chimeras for cell surface proteins uses the HA signal and membrane anchor. The resulting expressed proteins are, like those of Example 5, carried to the surface of the infected cells and presented for immunogenic response.

20 Referring to Figures 7 and 9, the HA gene insert has a blunt-end generating HincII (SalI) restriction site between codons 16 and 17 and a blunt-end generating PvuII restriction site between codons 450 and 451. Cleavage of the HA gene with HincII (SalI) and PvuII

25 excises the major portion of the HA protein and permits insertion of the hormone derived monomer and multimers between the signal sequence and the membrane anchor. The HA gene as inserted into pHA2 or pHA3 is used, as this vector does not contain interfering restriction

30 sites.

The preparation of the analogous vectors containing the LH sequences is as follows. For bovine LH, pHA3 is digested with EcoRI which cuts at codons 226-227 and 394-395 of the HA gene and upon blunting

-60-

leaves a two base pair extension beyond the codon boundary at either end. The bovine LH sequence containing, if desired, the multimeric forms is digested with DdeI which cuts between codons 18 and 19 and
 5 between 86 and 87 as shown in Figure 3 and which leaves an additional base pair extending beyond the codon boundary at either end. Therefore, ligation of the bLH sequences into the vector prepared as above places the bLH coding sequences into reading frame with those of
 10 the HA gene.

In a similar manner, pHA2 is digested with HincII (see Figure 7) which cleaves between codons 16 and 17 at the codon boundary and in codon 208 to leave one base pair extending beyond the codon boundary of
 15 codon 209. This permits ligation of the human monomeric or multimeric SLH DNA which is excised from the cloning vector by digestion with NcoI and StuI to obtain the portion spanning codon 1 through all but the last base pair of codon 115. This places the human SLH cDNA in
 20 reading frame with the HA sequences.

For dog β LH, the multimerized fragment from the pBR322 based vector of Example I is removed as a RsaI/SmaI blunted fragment and ligated into pHA2, which has been digested with SalI, blunted and then digested
 25 with HincII. The relevant junction regions are:

16	43	115	43	115	211
.....GTC GA	A CGAGTC CC A CGACTC CC	A ACC
HA blunt SalI	Rsa	Rsa-Sma multimer	Sma	HA HincII site #2	

30 Correct ligations are confirmed by restriction analysis. The desired immunogens are inserted in correct reading frame between the signal and membrane

-61-

anchor in a series of vectors designated pCTP_n/HAMB,
pHLH_n/HAMB, etc. or generically, pHormone/HAMB.

Example 7

5 Preparation of Intermediate Vectors Providing Chimeras for Secretion of Hormone

Immunogen Using HA Signal

 Vectors providing for secretion may be
constructed in three ways. First, the vectors
10 constructed in Example 5, which contain hormone antigen
in the antigenic regions of the HA carrier (Stu, Nru,
Hpa, RV) can be further modified to delete the membrane
anchor portion of the HA protein by using site-specific
mutagenesis for deletion of the codons downstream of
15 codon 514. Second, an NdeI/PvuII fragment between

codons 451 and 510 may be deleted using restriction
cleavage and religation for those vectors derived from
pHA2. Third, a portion of HA lacking the membrane
anchor can be used.

20 In the first approach, illustrated in Figure
~~10a, the entire coding sequence from pHormone/HA/Stu or~~
the analogous Nru, Hpa, or RV vectors is excised as a
BamHI/SmaI fragment and ligated into SmaI/BamHI-digested
M13 for mutagenesis using the primer

25

5'-GGGGTAAAATTGAGCTGATTGATTGACCCG-3'.

which deletes the membrane anchor portion downstream of
codon 504 as shown. The altered fragment is then
30 religated into the large fragment obtained from
BamHI/SmaI digestion of, for example, the starting,
unaltered vector, pHA2. The resulting vectors are
designated, generically, pHormone/HA₅₀₄/site.

-62-

In the second approach, shown in Figure 10b, vectors containing the monomer or multimer derived from pHA2 in Example 5, e.g. pCTP/HA/Stu or pGnRH/HA/RV, may be digested with PvuII and PstI to delete the desired region. The 3' overhang created by NdeI digestion is treated with Klenow in the presence of dCTP and the vectors are religated. Nucleotides 1601-1729 (Gething, et al, Nature (1980), supra) are deleted, fusing codon 450 to the last nucleotide of codon 511, and the translation proceeds in a new reading frame for another 8 codons and is then terminated by the third stop codon in the cloning vector. These vectors are designated, generically, pHormone/HA₄₅₀/site.

Still another anchor-minus vector is constructed from an anchor-minus form of the HA gene from pSVEH20-A (Gething, M.J., et al, Nature (1982) 300:598-603) as the carrier protein, as shown in Figure 10c. The HA minus anchor portion is obtained as a BamHI/NdeI fragment for insertion into pVA2 by converting the downstream BamHI site in this partial gene to NdeI. This is accomplished by digesting pSVEHA20 with BamHI, treating with Klenow and the four dNTPs, and ligation to an NdeI linker. Subsequent digestion with BalI and NdeI provides the appropriate BalI/NdeI fragment for insertion into pVA1, to obtain pVA1/HA₄₅₀-A-. pVA1/HA₄₅₀-A- is subsequently modified by mutagenesis to accommodate the desired Hormone at the antigenic sites in a manner precisely analogous to that described for pVA1/HA in Example 5.

Example 8

Other Intermediate Vectors with Signal Sequences

The signal sequences from other proteins may also be used to construct intermediate vectors so that

-63-

the ultimate recombinant vaccinia carrier will express hormone derived antigen as a secreted protein.

In one such construction, a synthetic gene encoding the bacterial alkaline phosphatase (BAP) signal sequence may be used. The construction of intermediate vectors carrying this sequence operably linked to multimer is shown in Figure 11.

The gene is synthesized using conventional techniques by construction of a series of complementary oligomers which hybridize to display compatible restriction sites, (ol5-o20) as illustrated in Figure 11. The synthetic gene fragment having a partial NcoI 5' overhang and HindIII 3' overhang is ligated into a host vector, pSS1, which has been digested with NcoI and HindIII. pSS1 is a pBR322 derivative in which the synthetic oligonucleotides ol3 and ol4 shown in Figure 11 are ligated into the BamHI/EcoRI vector fragment of pBR322 to create a series of convenient restriction sites. The resulting vector, pSS2, is digested with HindIII and blunt-ended with Klenow, and then ligated to blunt-ended hormone derived fragments using a tenfold molar excess of the insert. The resulting vectors, pHormone/BAP, then contain the hormone antigen in reading frame with the BAP signal sequence and terminated with a reading frame in-frame stop codon and bounded by SmaI and BamHI sites provided by the oligomer fragment which had been inserted in pBR322. These intermediate vectors, pHormone/BAP, are then used as sources of the BamHI/SmaI insert for transfer into vaccinia.

The preprorenin signal sequence linked to the hormone antigen fragment may also be used. This construction is shown in Figure 12. As shown, the pSS1 is digested with HindIII, blunted with Klenow, and

-64-

phenol extracted, digested with NcoI, and the vector fragment isolated. The blunted hormone antigen is ligated to the vector fragment along with the renin signal sequence. The renin signal sequence is obtained
5 as an NcoI/RsaI signal sequence-bearing fragment excised from pPP14. (pPP14 is extensively described in U.S. Serial No. 719,414, filed 3 April 1985, assigned to the same assignee and incorporated herein by reference; pPP14 carries the preprorenin cDNA insert). The
10 ligation products are the pHormone/renin series, where the renin signal sequence is placed immediately upstream of the hormone antigen encoding gene, and the vector donates an in-frame stop codon (and two unimportant extra amino acids) immediately downstream of the hormone
15 gene.

Example 9

Preparation of Vaccinia Recombinants

All of the intermediate vectors whose
20 preparation has been described in Examples 3, 5-7, and 8 contain hormone antigen bearing cassettes which can be excised as BamHI/SmaI fragments for insertion into pGS20 by digesting the intermediate vectors with BamHI and SmaI, isolating the excised fragments on gels, and
25 treating BamHI/SmaI-digested pGS20 with a tenfold molar excess of the isolated fragment. The resulting pGS20 derivatives contain the hormone antigen cassette in operable linkage to the vaccinia promoter and bounded by the portions of the TK gene, as shown in Figure 13.

30 These recombinant vectors are transfected into cells infected with wild-type vaccinia virus, which was purchased from Wyeth Laboratories, Inc. (Marietta, PA) and plaque purified twice in CV-1 cells. A small aliquot of virus stock (0.1 ml) is diluted with an equal

-65-

volume of trypsin (0.25 mg/ml) and incubated for 30 min at 37°C with vortexing, followed by sonication to disperse any cell clumps. The virus is diluted to a concentration of 5×10^4 plaque-forming units per ml in phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and penicillin/streptomycin. CV-1 cells are infected in monolayers on 60 mm plates with 1 ml vaccinia virus to give a multiplicity of infection of 0.05 pfu/cell. The virus inoculum is incubated on the cells for 2 hr at 37°C with rocking.

The DNA for transformation is prepared as described in Graham, et al, Virology (1973) 52:456; Stow, et al, J Gen Virol (1976) 33:447; and Frost, et al, Virology (1978) 91:39. Briefly, 5-10 µg of plasmid-(pGS20-derivative)-DNA, and 1-2 µg wild-type vaccinia virus DNA are added to 1 ml to Hepes-buffered saline (0.14 molar NaCl, 5 mM KCl, 1 mM Na phosphate, 0.1% dextrose, 20 mM Hepes, pH 7.05), and 50 µl of 2.5 molar CaCl_2 is added. The solutions are mixed and left at room temperature for 30 min, and the desired precipitate of DNA forms in this time.

The virus inoculum is aspirated from the CV-1 cell layers, and 1 ml of the DNA precipitate is substituted and the layers left at room temperature for 30 min, after which 9 ml prewarmed Eagle's MEM containing 8% FBS is added. The layers are incubated for 3.5 hr at 37°C, before aspirating off the medium and replacing it with 10 ml fresh Eagle's MEM containing 8% FBS. The monolayers are then left at 37°C for 2 days, resulting in the development of vaccinia cytopathic effects. The cells and virus are harvested by scraping, spun down, and resuspended in 0.5 ml Eagle's MEM and frozen at -20°C.

-66-

The resuspended virus/cell pellets are freeze-thawed three times by freezing the cells at -20°C for 30 minutes and quickly thawing them at 37°C, followed by sonication for one minute to disperse the virus/cell clumps. The resulting crude virus stock is then inoculated in serial 10-fold dilutions onto 143 cells (Mackett, M., et al, J Virol (1984) 49:857-864), a human tk- cell line. After two hours at 37°C, the 143 monolayers are then overlaid with 1% agarose containing 1 x modified Eagle's medium, 5% fetal bovine serum, and 25 µg/ml 5-bromodeoxyuridine (BUdR). After incubation for two days at 37°C, BUdR-resistant plaques are picked and grown for 48 hours at 37°C in 24 well plates of 143 cell monolayers in the presence of 25 µg/ml BUdR.

Virus is harvested and assayed for the presence of the hormone antigen gene by DNA-DNA dot blot hybridization, as follows: cells are scraped from the dish into an Eppendorf centrifuge tube, centrifuged for 1 minute, and the cell pellet resuspended in 0.2 ml PBS. After freeze-thawing 3 times and sonicating as described above, the sonicate is applied to a nitrocellulose filter and air dried. A wild-type virus control is also spotted onto the same filter. The filter is then placed on paper soaked in (1) 0.5 M NaOH, (2) 1 M Tris-HCl, pH 7.5, and (3) 2 x SSC, for 5 minutes each. The filter is then baked at 80°C under vacuum for 2 hours. The baked filter is prehybridized for 1 hour at 42°C in 5 ml 50% formamide, 4 x SSC, 5 x Denhardt's solution, and 0.1 mg/ml sheared and boiled salmon sperm DNA. The filter is then hybridized overnight at 42°C to 2 x 10⁷ cpm of ³²P-labeled probe containing the desired hormone, for example, the hCG CTF gene probe; in 5 ml prehybridization buffer with 10 mg/ml dextran sulfate added. The probe is prepared by isolating 5

-67-

µg of hCG CTP gene insert from one of the hCG CTP gene intermediate vectors and nick translating the DNA using a commercially available nick translation kit. The filter is then washed twice for 30 minutes in 0.5 x SSC. 5 0.1% SDS, air dried, and autoradiographed overnight at -70°C.

Virus containing desired hormone gene inserts are infected onto monolayers of 143 cells and left until a confluent cytopathic effect is obtained. The culture 10 medium is then aspirated off and the cells lysed in 1% SDS, 0.1 M β-mercaptoethanol, 50 mM Tris-HCl, pH 7.8. The lysate is made 0.5 mg/ml in proteinase K, incubated 4 hours at 37°C, phenol extracted, ethanol precipitated, and analyzed by restriction enzyme analysis to show that 15 the vaccinia genomes contain the desired genes.

Example 10

Bioassay

Rabbits are used as subjects to assess the 20 ability of the recombinant vaccinia to raise titers of neutralizing anti-hormone antibodies in serum. The following describes the procedure for CTP encoding vaccines; however, other hormones can be assayed using, for example, GnRH, LH, or FSH in place of CTP. The 25 procedure is analogous, but the antigen component in each assay is the appropriate hormone.

Pairs of rabbits are inoculated with wild-type or recombinant virus by intradermal injection of $1-2 \times 10^8$ plaque-forming units distributed in 2-3 sites on 30 the back. Rabbits are bled from their ears at days 0, 14, 28, and sera tested for the presence of anti-hCG neutralizing antibodies in a radioligand receptor assay as follows.

-68-

Commercially available hCG is iodinated in a 100 µl reaction by the chloramine T method of Greenwood, F.C., et al (J Biochem (1963) 89:114-123) in a 100 µl reaction mixture containing 5 µg hCG, 1 mCi Na¹²⁵I, 0.1 µg chloramine T, and 0.1 M NaPO₄, pH 7.5. The mixture is incubated at room temperature for 10 minutes and the reaction stopped with the addition of 0.1 µg sodium metabisulfite. Purification of ¹²⁵I-hCG is performed by reverse-phase HPLC on a C₁₈ column.

The interstitial cells of rat testis are homogenized in phosphate-buffered saline (PBS) as described in Catt, K.J., et al (J Clin Endocrinol Metab (1974) 34:123-127). The homogenate is centrifuged at 1500 g for 50 min and the pellet resuspended in 10 ml PBS per testis. A saturation binding curve is performed by incubating 100 µl of testis homogenate, 50 µl of tracer I¹²⁵-hCG (20,000 cpm) in PBS-bovine gamma globulin (1 mg/ml), and 100 µl of hCG at various concentrations 0.1 to 100 ng/ml in preimmune serum, including a no-hCG control. After incubation at 24°C for 18 hours, the receptor-bound tracer is quantified by filtration through albumin-soaked 0.45 µ cellulose membranes and counted in a Beckman Gamma 5000 gamma counter. The concentration of hCG that results in 80% binding to the testis homogenate is then used with the labeled tracer in subsequent assays. Sera are tested for neutralizing activity by incubation with 100 µl testis homogenate, 50 µl of tracer I¹²⁵-hCG (20,000 cpm) in PBS-bovine gamma globulin (1 mg/ml), 50 µl of hCG at the concentration determined in the saturation binding assay above, and 50 µl of sera diluted from 1:10 to 1:5000, including a no-serum control. The samples are then incubated, and bound tracer isolated

-69-

and counted, exactly as described for the saturation binding assay above.

5 The titer of neutralizing activity is the dilution of serum that inhibits 50% of the binding in this assay. The titer of sera from animals that have low-level immunoneutralization responses will be 1:5 to 1:50, moderate responders will have sera with titers of 1:50 to 1:250, and strong responders will have sera with titers of greater than 1:1000. Recombinant vaccinia
10 directing the expression of intracellular hCG antigens are more likely to produce low responses, and various secreted and membrane-bound antigens are more likely to produce moderate and strong responses. An alternate assay based on the change in weight of rat uterus can
15 also be used.

The recombinant vaccinia which produce moderate to strong immunoneutralization responses in rabbits or show positive responses in the rat uterus weight assay will be tested for fertility control in baboons or
20 rhesus monkeys. In general, the recombinant viruses that produce strong responses in rabbits will confer protection for longer periods of time, up to 3-5 years or more in humans, and the recombinant viruses that produce moderate responses in rabbits will protect for a
25 shorter period of time, from 6 months to 3 years in humans.

Example 11

Preparation of Peptide Vaccines

30 The coding sequences for the autoantigens described above may also be expressed using conventional recombinant techniques, and the resulting peptides used as conventional vaccines. In one approach, the constructions in pGS20 which have been described in

-70-

detail can be transiently expressed in CV-1 monkey cells using vaccinia virus coinfection, in the procedure of Cochran, M.A., et al, Proc Natl Acad Sci USA (1985) 82:19-23. Briefly, the CV-1 cells are infected with
5 vaccinia at a moi of 30, and then transfected using calcium phosphate precipitation with the autoantigen-containing pGS20. The cells are harvested 12-48 hours after transfection, and the autoantigen recovered. If the autoantigen is membrane bound, the
10 cells are solubilized; if secreted, the autoantigen is recovered from the medium.

In alternate approaches, the coding sequences for the hormones, either as multimers, fusion proteins with secondary carrier, or combinations thereof, are
15 placed under control of conventional control sequences in expression vectors. For procaryotic expression, a particularly useful host vector is pKT52. pKT52 contains the "trc" promoter followed by an ATG and is constructed as follows below. The "trc" promoter
20 contains the upstream portions of the trp promoter and the downstream, operator-containing, regions of the lac promoter.

To prepare pKT52, pKK233-2 (Amann, E., et al, Gene (1985) 40:183-190) was digested with EcoRI and
25 PvuII, filled in with dATP and dTTP, and religated to obtain the correct construction pKT52.

pKT52 contains the desired trc promoter, a downstream ATG start codon, and downstream NcoI, PstI and HindIII sites. The autoantigen containing cassette
30 can be excised from any of the vectors set forth above and inserted into NcoI/HindIII digested pKT52.

For eucaryotic expression, a useful host vector is pMT, which contains a convenient EcoRI site, and its

-71-

related vector pMT-Apo. These vectors are constructed as follows.

The host vector, pMT contains the metallothionein II (hMTII) control sequences, as
5 described by Karin, M., et al, Nature (1982) 299:797-802. It is obtained by ligating the promoter into pUC8 as follows.

Plasmid 84H (Karin, M., et al (supra)) which carries the hMTII gene was digested to completion with
10 BamHI, treated with exonuclease Bal-31 to remove terminal nucleotides, and then digested with HindIII to liberate an 840 bp fragment containing nucleotides -765 to +70 of the hMTII gene (nucleotide +1 is the first nucleotide transcribed). The 840 bp fragment was
15 isolated and ligated with HindIII/HincII digested pUC8 (Vieira, J., et al, Gene (1982) 19:259-268) and the ligation mixture transformed into E. coli MC1061. The correct construction of pMT was confirmed by dideoxy nucleotide sequencing.

20 In addition, a derivative of the pMT, pMT-Apo, containing C-terminal regulatory signals was also prepared. pMT-Apo harbors a portion of the human liver protein ApoA₁ gene (Shoulders, C. C., et al, Nucleic Acids Res (1983) 11:2827-2837) which contains the
25 3'-terminal regulatory signals. A PstI/PstI 2.2 kb fragment of ApoA₁ gene (blunt ended) was cloned into the SmaI site of the pMT polylinker region, and the majority of the ApoA₁ gene removed by digestion with BamHI, blunt ending with Klenow, digestion with StuI,
30 and religation. The resulting vector contains roughly 500 bp of the ApoA₁ gene from the 3' terminus as confirmed by dideoxy-sequence analysis. The autoantigen is inserted as an NcoI/EcoRI fragment.

-72-

Example 12Antigenicity of Recombinantly Produced Peptides

Intermediate vectors containing multimers of 2 copies of ser110 CTP, 2 copies of des110 CTP, or 3 copies of GnRH between the HincII and PvuII sites of the HA gene of pHA2 were constructed using the appropriate multimer as described in Example 6, to give pCTP₂(ser110)/HAMB, pCTP₂(nol10)/HAMB, and pGnRH₃/HAMB, respectively. The BamHI/SmaI fragments containing the coding sequences for amino acids 1-16 and 451-551 of the HA gene bracketing the specified multimers were transferred to pGS20 as described above, and the resulting pGS20/Carrier/Hormone vectors transformed into vaccinia infected CV-1 cells as in Example 11. The proteins were labeled with ³⁵S methionine, recovered from the cell surfaces, and a portion of them immunoprecipitated with polyclonal sera raised against hCG or GnRH as appropriate. The labeled proteins and their immunoprecipitates were analyzed on a 12.5% Laemmli gel, and the results are shown in Figure 14.

Lanes 1 and 14 of Figure 14 show low and high molecular weight standard, lanes 2-5 are total proteins from cells transformed with the vectors indicated on the figure. The high background levels did not permit proteins uniquely produced by the inserts to become apparent. However, immunoprecipitation with the appropriate antiserum permitted detection and verification of the molecular weights of these proteins.

Lanes 6 and 7 show the results of immunoprecipitation of membrane bound proteins of the CV-1 cells using anti-GnRH; lane 6 represents the pGS20/HA/GnRH₅ transformant, lane 7 is the pGS20 transformed control. The anti-GnRH is specific for the

-73-

GnRH mid-region, as described by King, J.A., et al, Endocrinology (1980) 106:707-717. The smeared band at approximately 23 kd is of roughly the correct molecular weight (17 kd contributed by HA, 6 kd by the multimer) and may be a glycosylated protein (there are two glycosylation sites - both in the HA sequence).

Lanes 8-13 are immunoprecipitates with Miles antiserum (lanes 11-13) or Meloy antiserum (lanes 8-10), both raised against whole hCG in rabbits. Lanes 10 and 13 represent pGS20/HA/CTP₂(ser110) transformants and show the presence of a new immunoprecipitated protein of approximately correct molecular weight (17 kd from the HA, 12 kd from the multimer). The control lanes 8 and 11, and the transformants with the nol10 multimer, lanes 9 and 12, do not show new bands.

Similar assays conducted on lysates of CV-1 cells infected with a recombinant vaccinia virus containing the DNA encoding the CTP 2-mer showed a band of correct molecular weight which specifically immunoprecipitated with anti-hCG antibody.

Example 13

Construction of Species Specific Vaccines

Advantage is taken of the regions of low species homology in the LH sequences shown in Figure 15 to construct peptides based on these which are species-specific antifertility vaccines. Appropriate oligomers encoding the peptides represented by amino acids 58-69, 104-118 of the bovine sequence and 58-68 and 104-107 of the dog sequence, as shown in Figure 15 represent positions of low homology with human LH, thus permitting vaccines made from them to be handled safely by humans.

-74-

These oligomers may be polymerized and blunted to obtain multimers which are treated as described above to generate vaccines. As the peptides are relatively short, it may be advantageous to use them simultaneously to obtain a vaccine of maximum effectiveness.

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-75-

Claims

1. A vaccine effective in raising antibodies against a target indigenous protein which comprises a
5 recombinantly produced immunogenic form of said protein, and wherein said immunogenic form is selected from the group consisting of:
 - a) a multimer which consists essentially of at least 2 repeating units of at least one epitope of said
10 indigenous protein.
 - b) at least one epitope of said indigenous protein conjugated to a non-bacterial polypeptide, and
 - c) a multimer which consists essentially of at least 2 repeating units of at least one epitope of said
15 indigenous protein conjugated to an additional polypeptide sequence.
2. A DNA sequence which encodes the immunogenic form protein of claim 1.
20
3. A recombinant host cell which is transformed with the DNA of claim 2.
4. The DNA sequence of claim 2 which is
25 disposed in a nonessential region of vaccinia virus.
5. The vaccine of claim 1 wherein the immunogenic form comprises a multimer which consists essentially of at least 2 repeating units of at least
30 one epitope of said indigenous protein.

-76-

6. The vaccine of claim 1 wherein the non-bacterial polypeptide is an influenza hemagglutinin.

5 7. The vaccine of claim 1 which is an antifertility vaccine effective in mammals.

8. The vaccine of claim 7 wherein the indigenous protein is selected from the group consisting of LH, GnRH, CG, and FSH.

9. A vaccine effective against mammalian fertility which comprises:

15 a vaccinia virus genome having disposed, in a nonessential region thereof, a DNA sequence having the formula Hormone_n, wherein n is an integer equal to 1-20 and Hormone represents a DNA sequence derived from the sequence encoding a reproductive hormone.

20 10. The vaccine of claim 9 wherein the DNA ~~sequence derived from the sequence encoding a~~ reproductive hormone is selected from those derived from the group consisting of:

25 CTP where CTP represents a DNA encoding the carboxy terminal portion (CTP) of the β -peptide of human chorionic gonadotropin (β -hCG);

GnRH where GnRH represents a DNA encoding a peptide derived from gonadotropin releasing hormone;

30 hLH where hLH represents a DNA encoding a peptide derived from human luteinizing hormone;

bLH where bLH represents a DNA encoding a peptide derived from bovine luteinizing hormone;

dLH where dLH represents a DNA encoding a peptide derived from dog luteinizing hormone;

-77-

cLH where cLH represents a DNA encoding a peptide derived from cat luteinizing hormone;

oLH where oLH represents a DNA encoding a peptide derived from ovine luteinizing hormone; and

5 FSH where FSH represents a DNA encoding a peptide derived from follicle stimulating hormone.

11. The vaccine of claim 10 wherein the DNA is selected from the group consisting of:

10 that which encodes the peptide consisting of amino acids 109-145 of β -hCG;

that which encodes the des₁₁₀ form of the peptide consisting of amino acids 109-145 of β -hCG;

15 that which encodes the ser₁₁₀ form of the peptide which is amino acids 109-145 of β -hCG;

that which encodes a peptide having the amino acid sequence of chicken II or human GnRH with a glutamine residue at the N-terminus;

20 that which encodes a peptide having the amino acid sequence of amino acids 2-111 of human LH;

that which encodes a peptide having the amino acid sequence of amino acids 22-85 of bovine LH; and

25 that which encodes a peptide having the amino acid sequence of amino acids 43-115 of dog LH.

12. The vaccine of claim 9 wherein the DNA of the formula Hormone_n is operably linked to a signal sequence capable of effecting the secretion of a protein comprising Hormone_n.

30

13. The vaccine of claim 9 wherein the DNA of the formula Hormone_n is disposed in an antigen-encoding portion of a DNA encoding HA.

-78-

14. A DNA sequence encoding a peptide
effective in controlling mammalian fertility which
comprises a DNA sequence encoding influenza
hemagglutinin (HA) having disposed therein DNA of the
5 formula Hormone_n.

15. Antibodies or the Fab portions thereof
raised against the vaccine of claim 1.

10

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20

25

30

1719

native

109 Thr Cys Asp Asp Pro Arg Phe Gln
ACC TGT GAT GAC CCC CGC TTC C
ACA CTA CTG GGG GCG AAG GT
BstNI

116

no110

109 111 Thr Asp Asp Pro Arg Phe Gln
ACC GAT GAC CCC CGC TTC C
CTA CTG GGG GCG AAG GT
BstNI BstNI

116

117 Asp Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu Pro
AG GAC TCC TCT TCC TCA AGG GCC CTT CCC CCC AGC CTT DCA
C CTG AGG AGA AGG AGT TTC CGG GGA GGG GGG TCG GAA GGT
BstNI BstNI

142 Ile Leu Pro Gln
GATC CTC CCA CAA
GAG GGT GTT TGG

ser110

109 110 111 Thr Ser Asp Asp Pro Arg Phe Gln
ACC TCT GAT GAC CCC CGC TTC C
AGA CTA CTG GGG GCG AAG GT
BstNI

116

Sau3A

141 Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro
AGT CCA TCC CGA CTC CCT GGG CCC TCG GAC ACC CC
TCA GGT AGG GCT GAG GGA CCC GGG AGC CTG TGG GG CTAG
Sau3A

no110 or ser110 A

B

C

TGG

BstNI

Sau3A

ACC

ACC

TGG

FIG. 1

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16

2/19

pig/cow/human: pyroGlu His Trp Ser Tyr Gly Leu Arg Pro Gly NH₂
 salmon: Trp Leu
 chicken I: Gln
 chicken II: His Trp Tyr

GnRH SEQUENCE FROM VARIOUS SPECIES

Human synthetic GnRH gene

Gln His Trp Ser Tyr Gly Leu Arg Pro Gly
 CAG CAC TGG TCC TAT GGA CTG CGC CCT GGA
 GTG ACC AGG ATA CCT GAC GCG GGA CCT GTC
 Gln overhang

Chicken II synthetic gene

Gln His Trp Ser His Gly Trp Tyr Pro Gly
 CAG CAC TGG TCC CAC GGA TGG TAT CCT GGA
 GTG ACC AGG GTG CCT ACC ATA GGA CCT GTC
 Gln overhang

GnRH GENES

FIG. 2

5 / 19

NUCLEOTIDE AND ENCODED AMINO ACID SEQUENCE OF HUMAN β LH

[illegible]

Scheme to construct multimers of a portion of the β peptide:

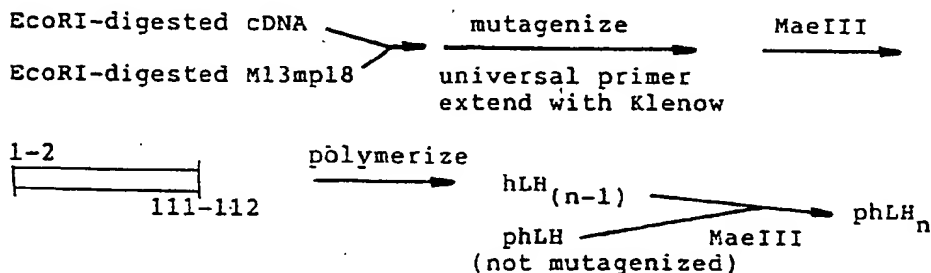


FIG. 4

6/19

Construction of Multimer Carrier Vectors

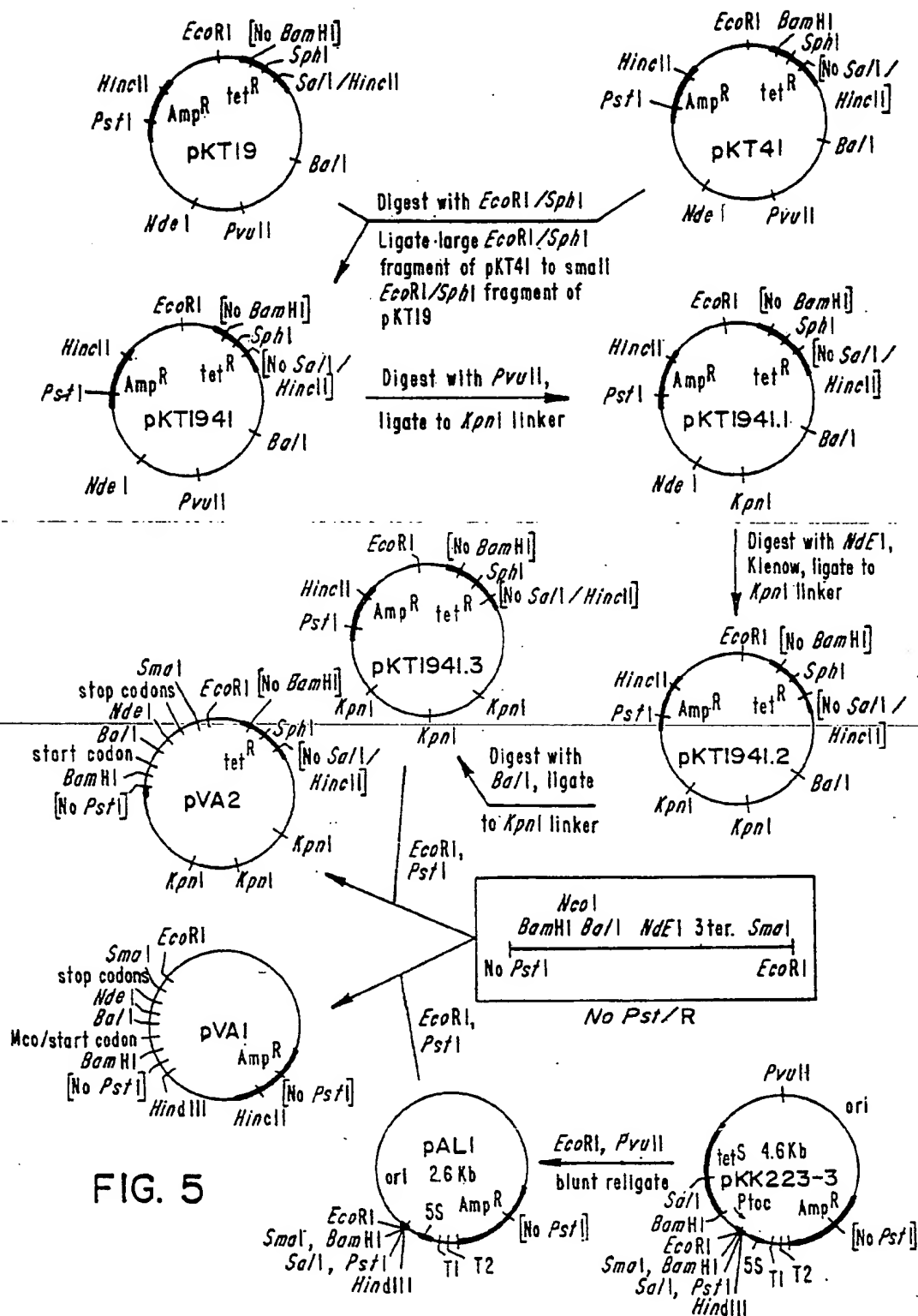


FIG. 5

7/19

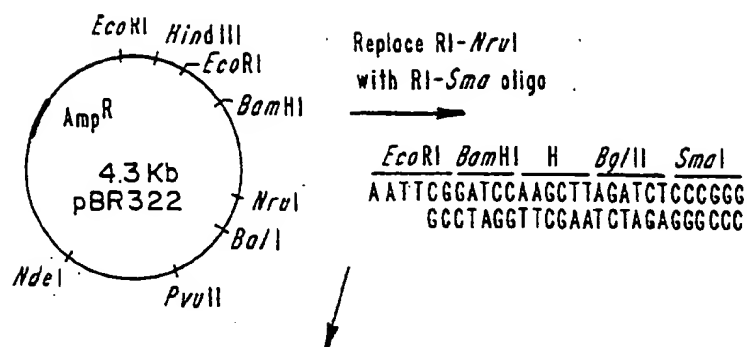
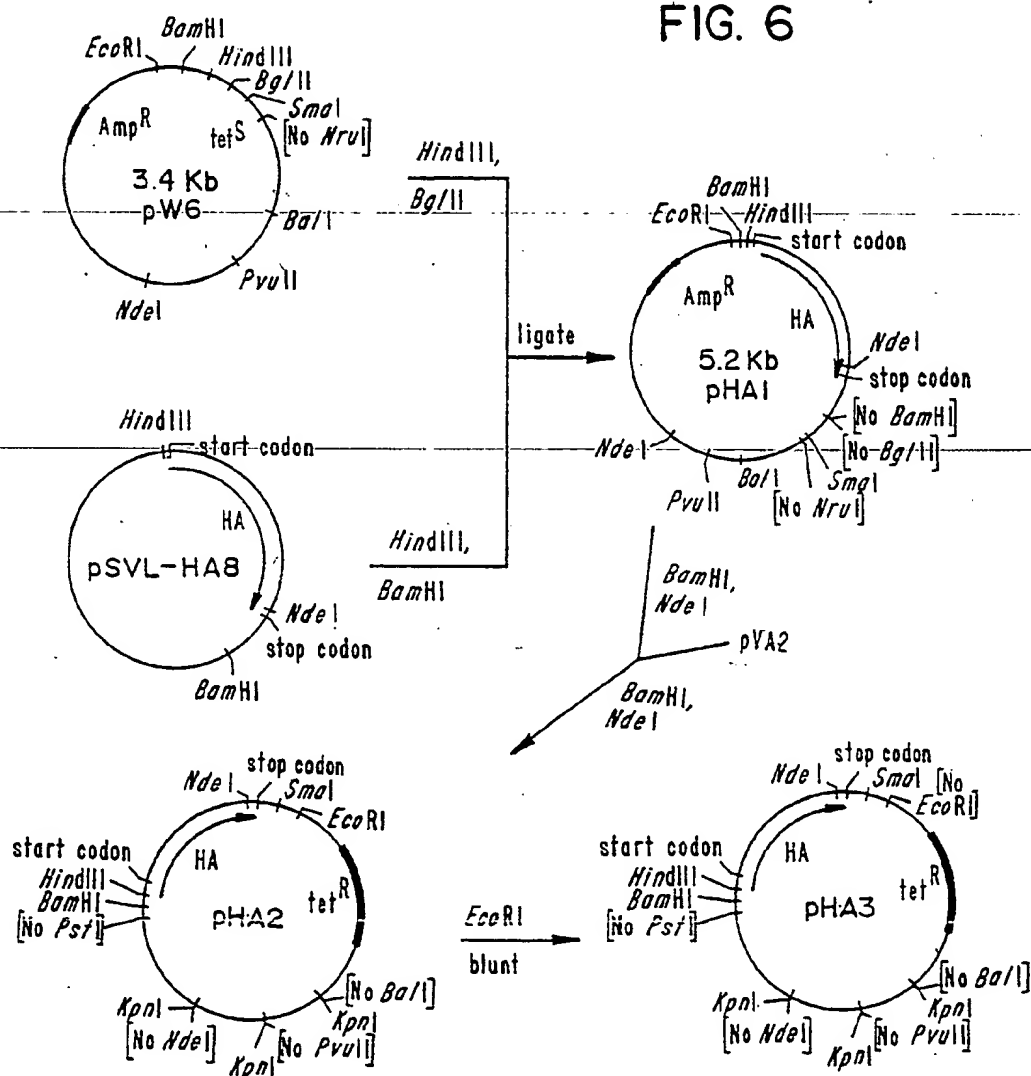
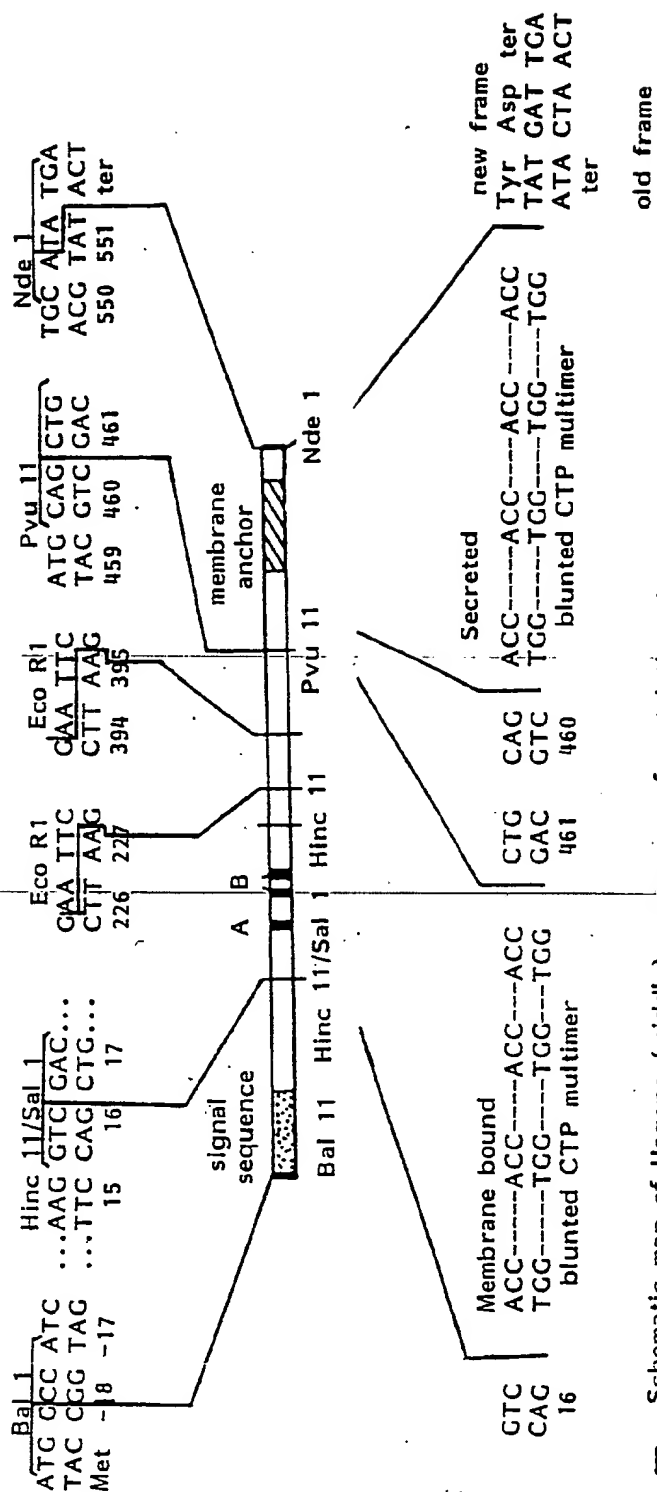


FIG. 6



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8 / 19



Schematic map of Hagene (middle), sequence of restriction sites important in cloning (above), and insertion site of membrane bound (bottom left) and secreting (bottom right) HA/hCG CTP fused polypeptides. The sequence of this antigenic variant, A/Japan/305/57 (H2) is from Gething, M.-J. et al., Nature 287, 301, 1980.

9/19

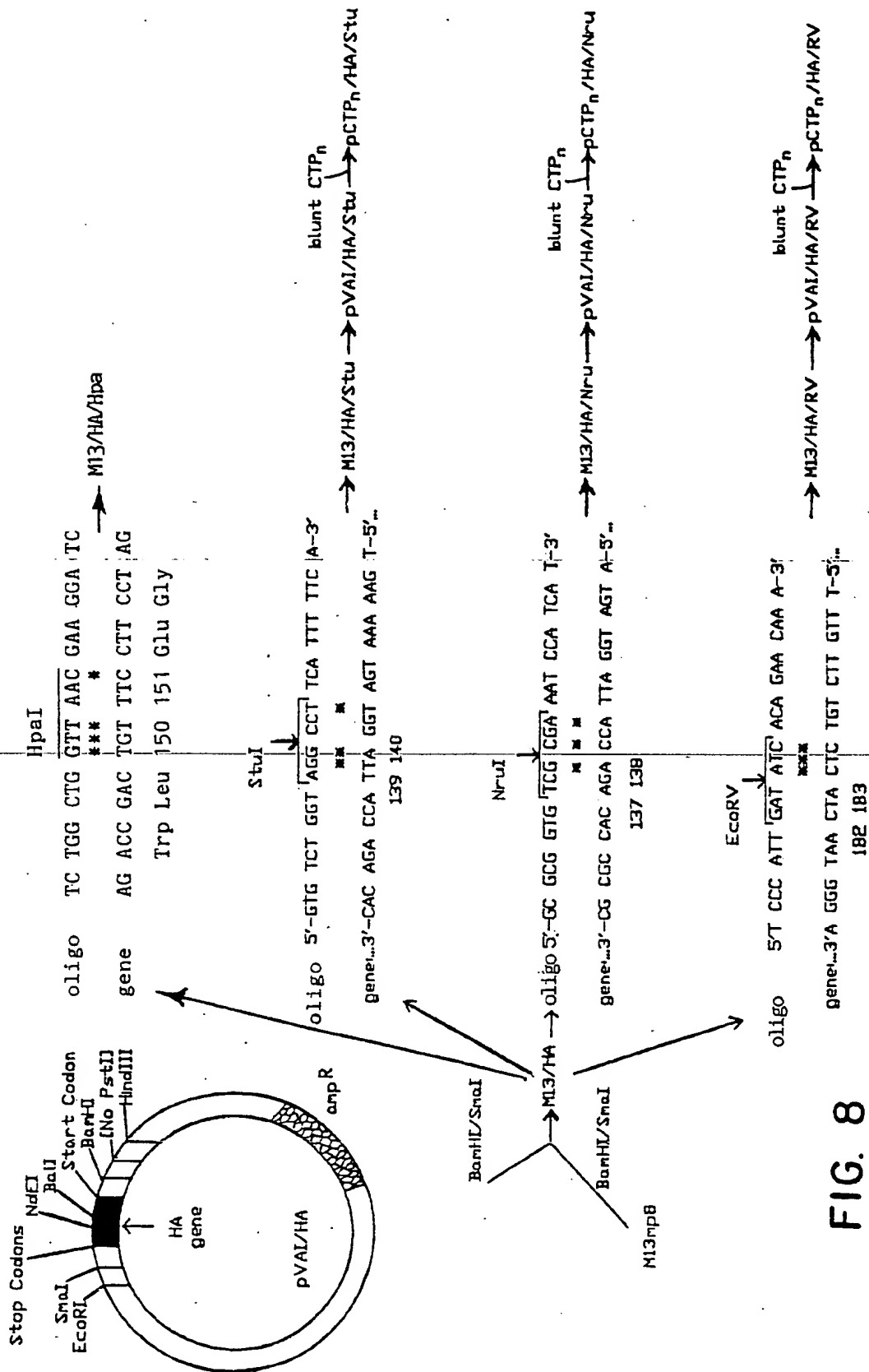


FIG. 8

10/19

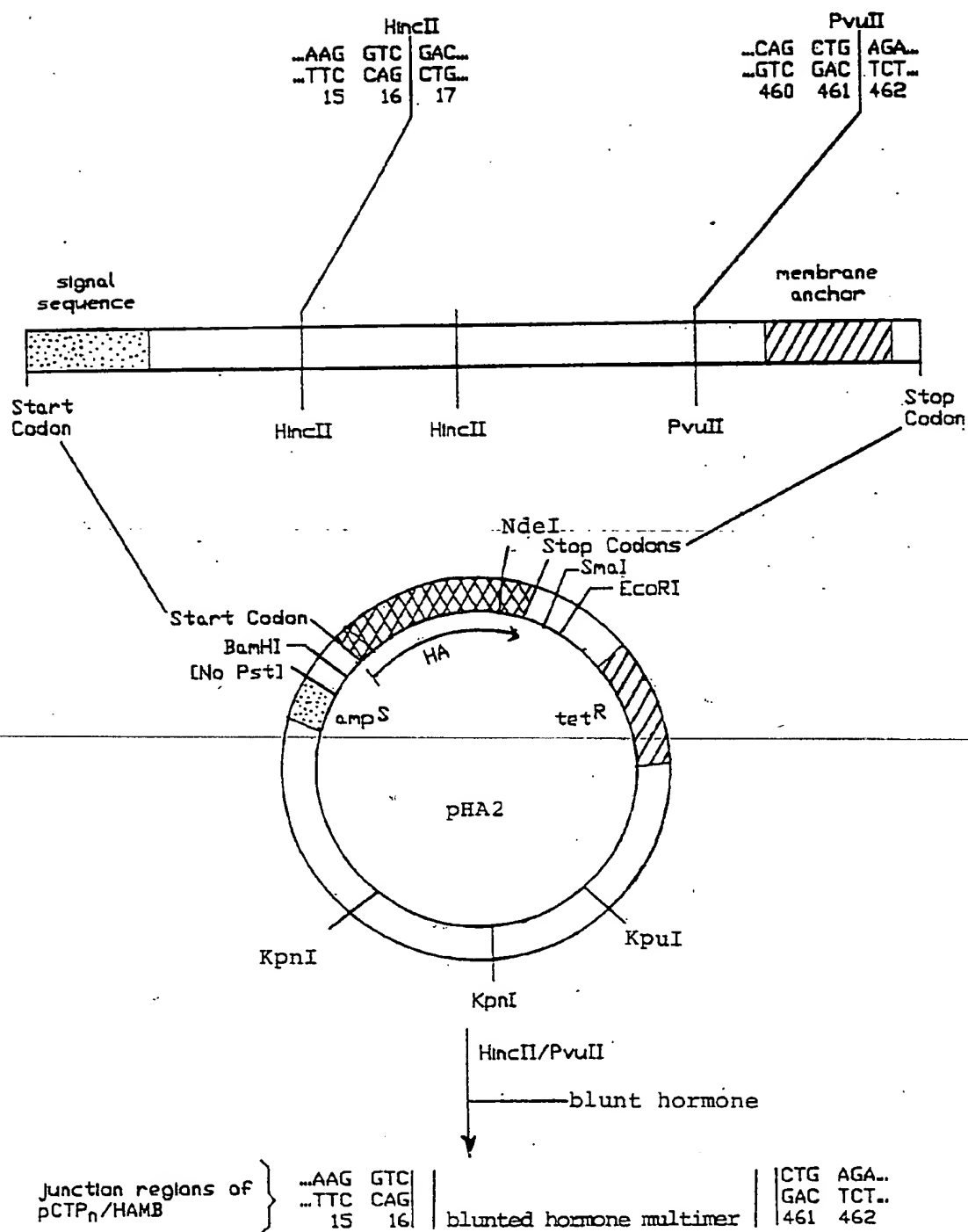


FIG. 9

SUBSTITUTE SHEET

11/19

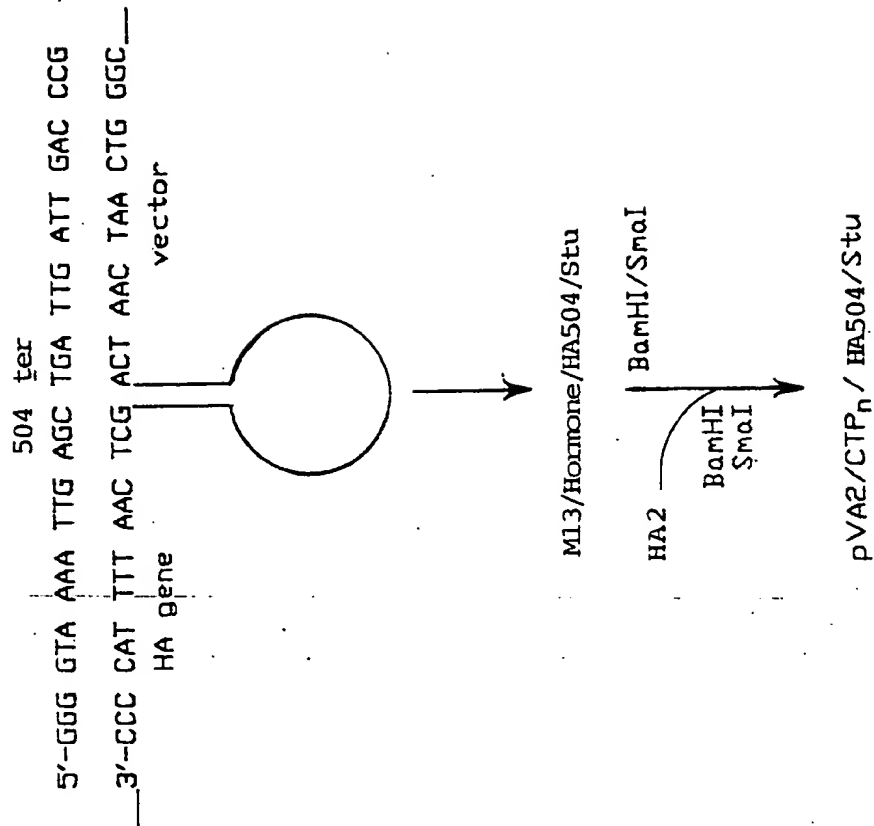


FIG. 10A

M13/CTP_n/HA/Stu
(see Figure 5)

0-12

gene in
HA vector

12/19

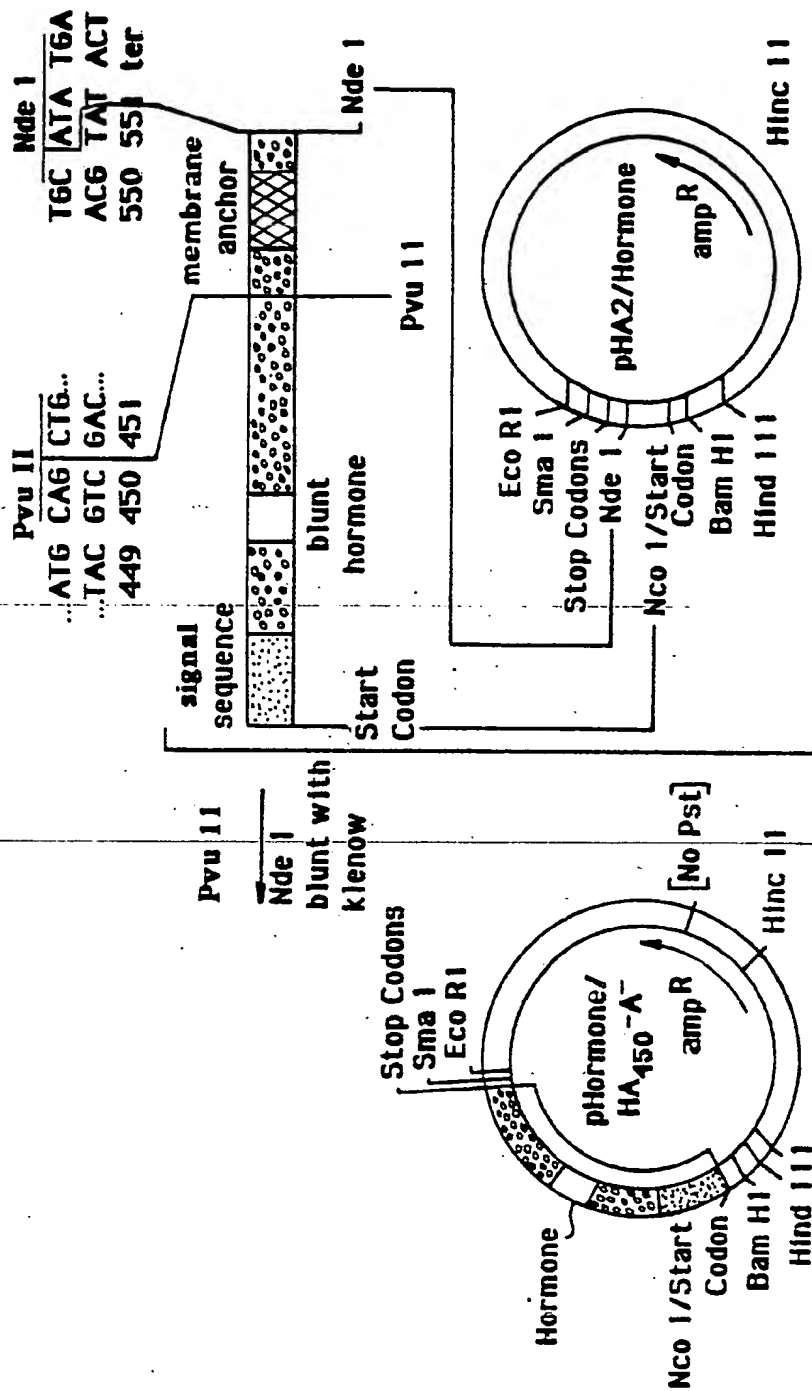


FIG. 10B

Junction regions of pHormone/HA₄₅₀-A⁻

ATG CAG	Hormone
TAC GTC	
449 450	

HA

13/19

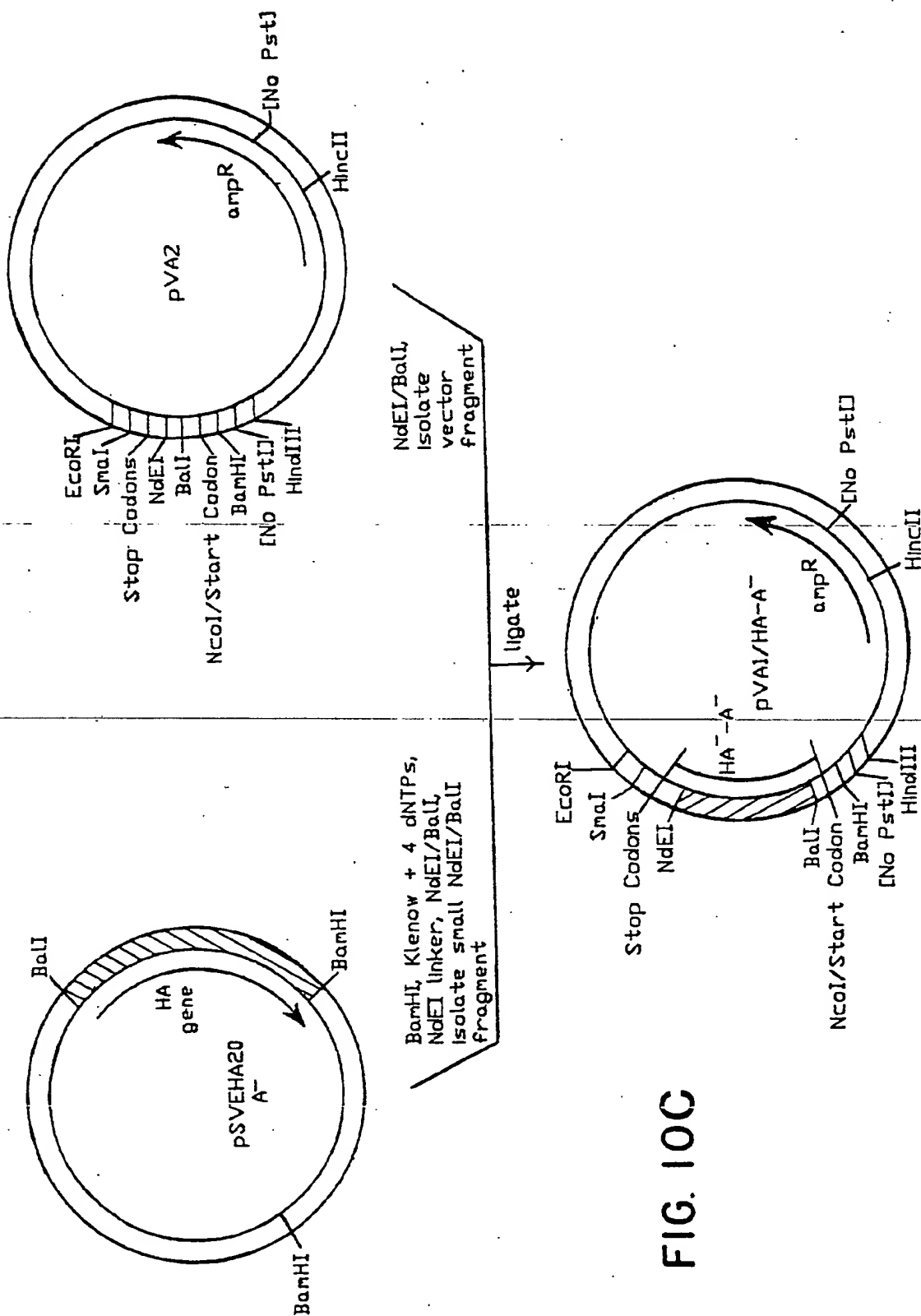


FIG. 10C

14/19

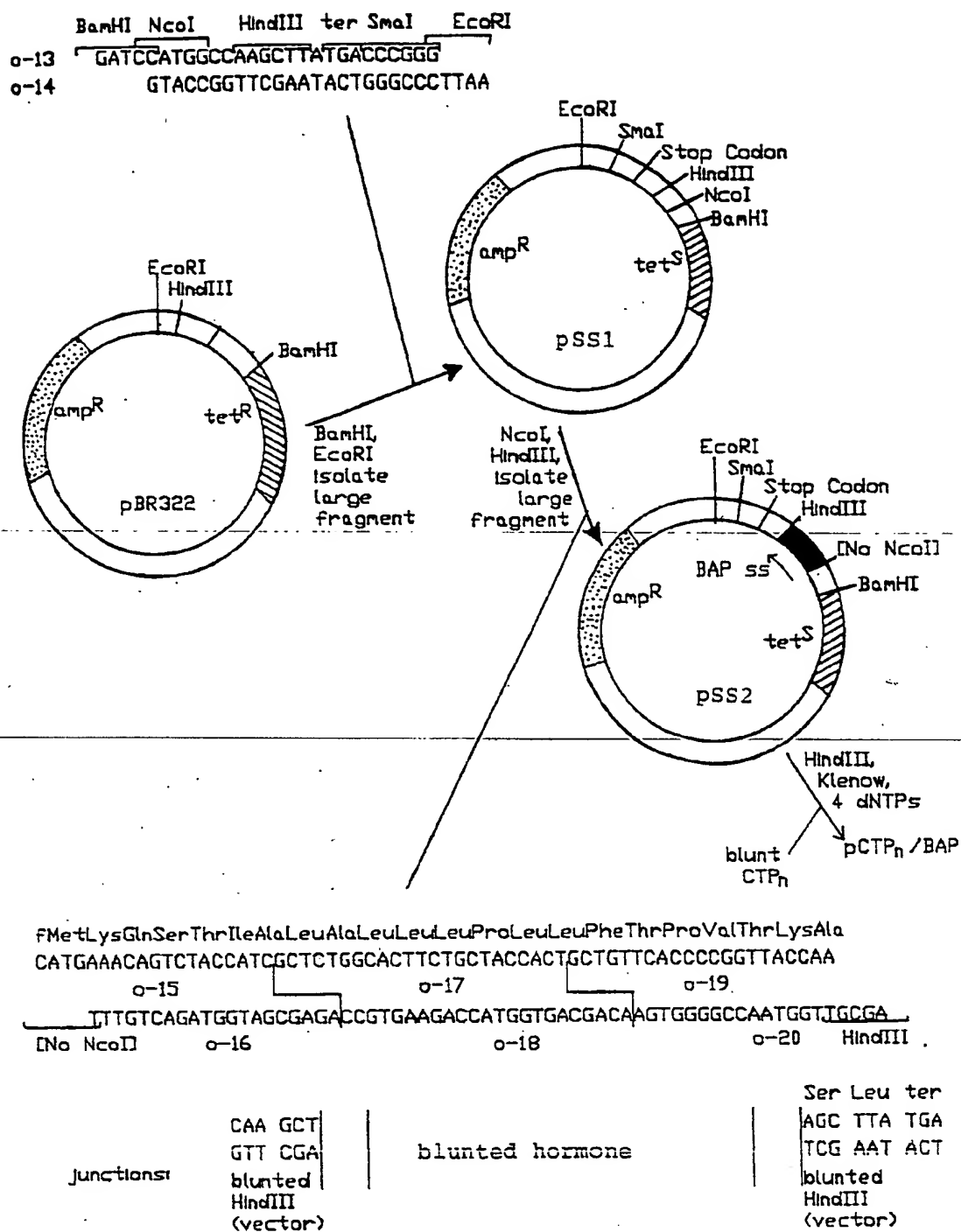


FIG. II

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15/19

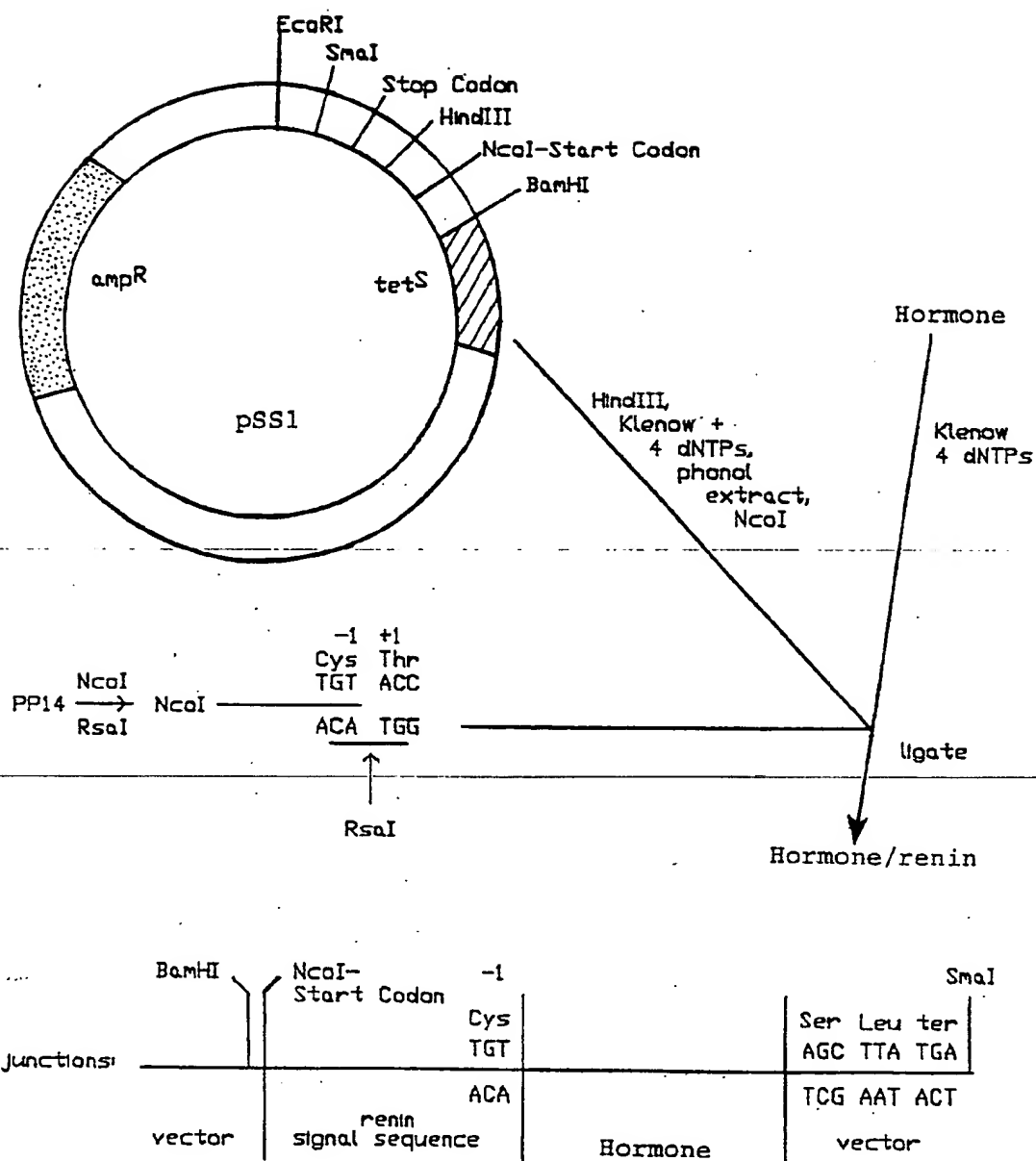
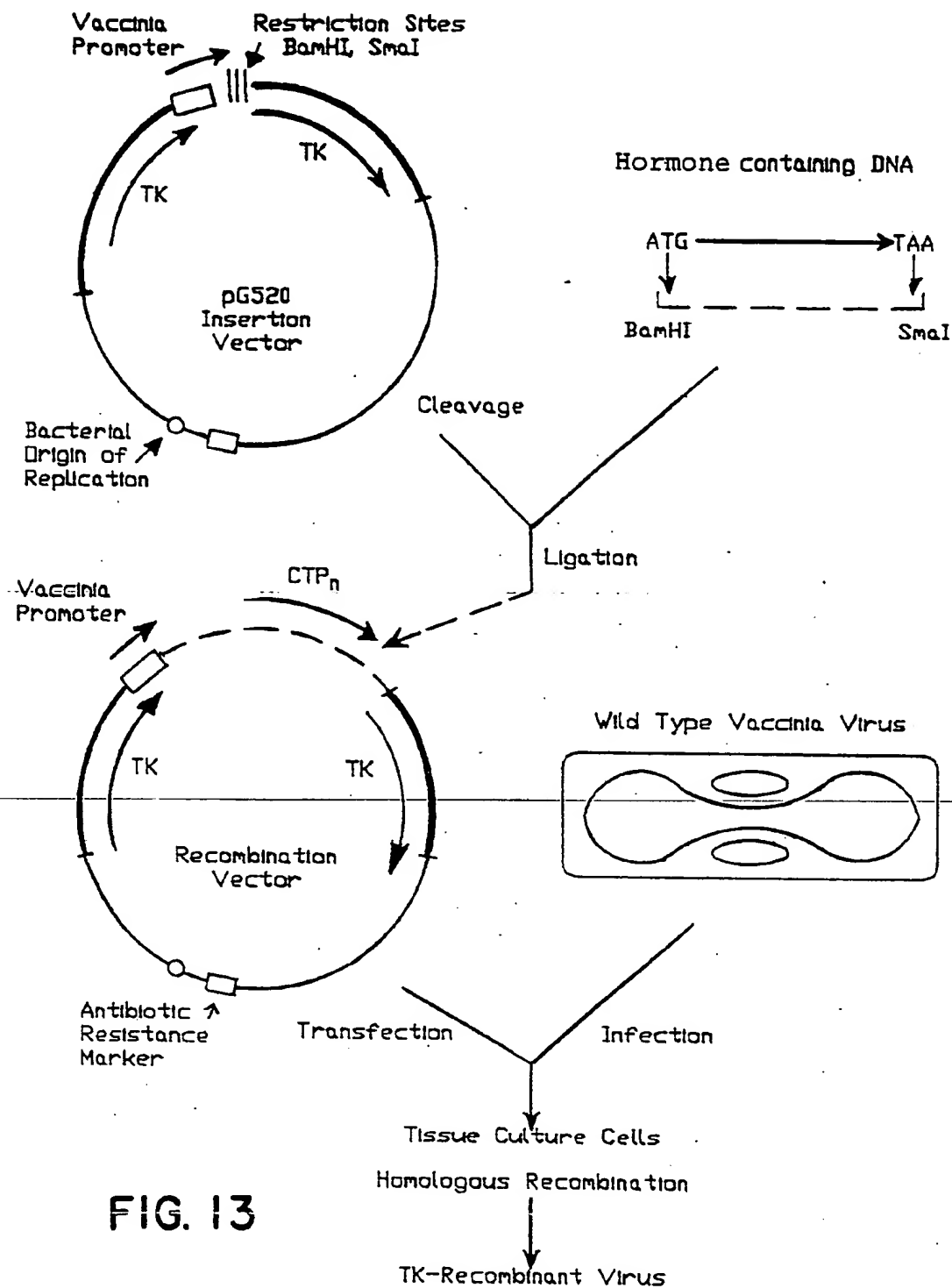


FIG. 12

SUBSTITUTE SHEET

16/19



SUBSTITUTE SHEET

17/19

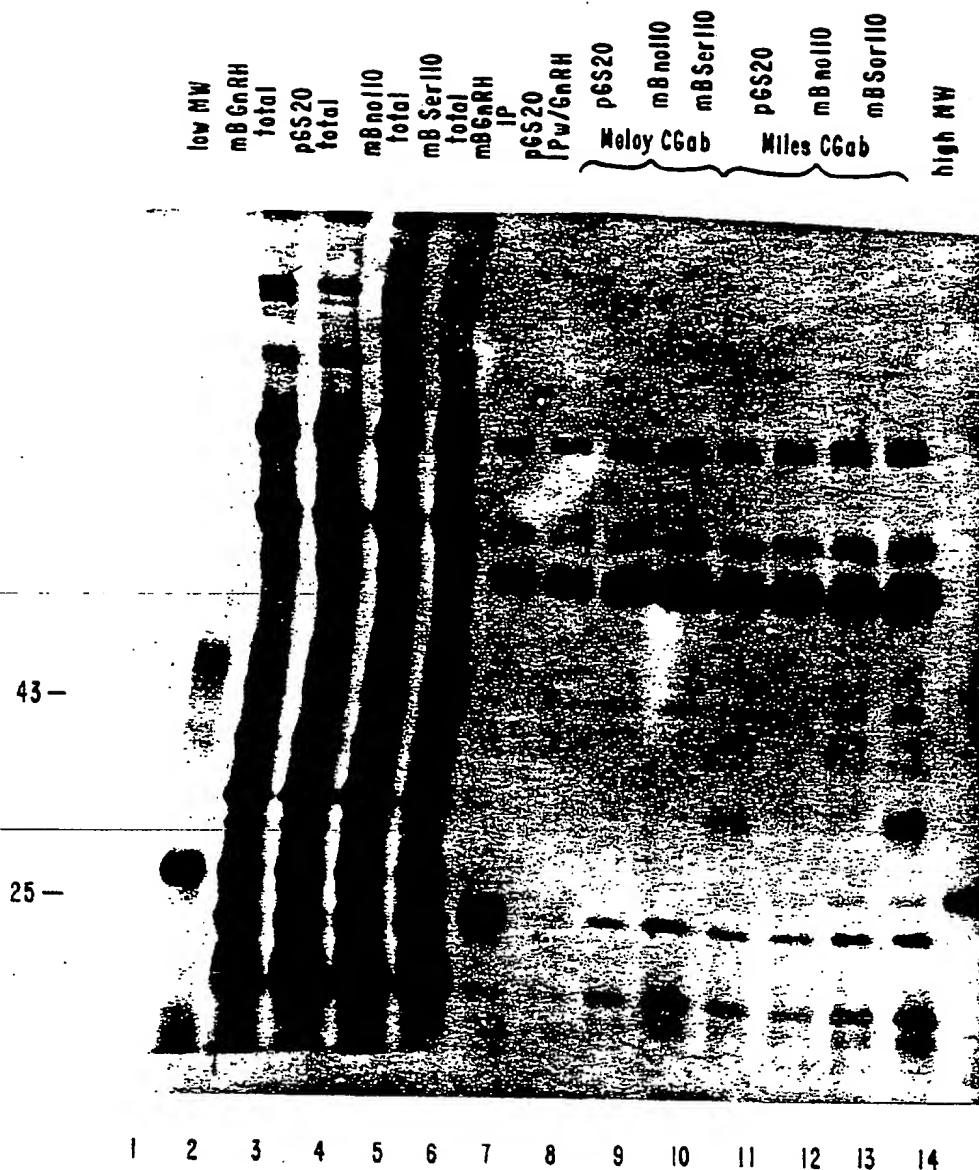


FIG. 14

COMPARISON OF HUMAN, RAT, COW, AND DOG SLH PROTEIN SEQUENCES

	His	Pro	Leu	Thr	Cys	Asp	His	Pro	Gln	Leu	Ser	Gly	Leu	Leu	120
Human	His	Pro	Leu	Thr	Cys	Asp	His	Pro	Gln	Leu	Ser	Gly	Leu	Leu	Phe Leu
Rat	Gln	Pro	Met	Thr	Cys	Asp	Leu	Pro	His	Leu	Pro	Gly	Leu	Leu	Leu Phe
Cow	Gln	Pro	Leu	Ala	Cys	Asp	His	Pro	Pro	Leu	Pro	Asp	Ile	Leu	Phe Leu
Dog	Gln	Ser	Leu	Ala	Cys	Asp	Arg	Pro	Leu	Leu	Pro	Gly	Leu	Leu	Phe Leu

SUBSTITUTE SHEET

19/19

COW-SPECIFIC AND DOG-SPECIFIC β LH PEPTIDES AND SYNTHETIC GENES

COW

cow β LH(58-69):

	*	*	*			*		*				
Thr	Tyr	His	Glu	Leu	Arg	Phe	Ala	Ser	Val	Arg	Leu	
ACC	TAC	CAT	GAG	CTG	CGC	TTC	GCC	TCC	GTT	CGG	CTC	
	ATG	GTA	CTC	GAC	GCG	AAG	CGG	AGG	CAA	GCC	GAG	TGG

[Ser110]-cow β LH(104-118):

	*	*	*		*				*		*	*	*	*	
Arg	Thr	Gln	Pro	Leu	Ala	Ser	Asp	His	Pro	Pro	Leu	Pro	Asp	Ile	
AGA	ACC	CAA	CCC	TTG	GCC	TCT	GAC	CAC	CCG	CCG	CTC	CCA	GAC	ATC	
	TGG	GTT	GGG	AAC	CGG	AGA	CAG	GTG	GGC	GGC	GAG	GGT	CTG	TAG	TCT

DOG

dog β LH(58-69):

	*	*	*	*		*					
Thr	Tyr	His	Glu	Leu	His	Phe	Ala	Ser	Ile	Arg	
ACC	TAC	CAT	GAG	CTG	CAC	TTT	GCT	TCA	ATC	CGG	
	ATG	GTA	GTC	GAC	GTG	AAA	CGA	AGT	TAG	GCC	TGG

[Ser110]-dog β LH(104-117):

	*	*	*	*		*		*		*		*		
Arg	Ala	Gln	Ser	Leu	Ala	Ser	Asp	Arg	Pro	Leu	Leu	Pro	Gly	
AGA	GCT	CAA	TCC	TTG	GCC	TCT	GAC	CGC	CCC	CTG	CTC	CCG	GGC	
	CGA	GTT	AGG	AAC	CGG	AGA	CTG	GCG	GGG	GAC	GAG	GGC	CCG	TCT

* = difference with the human sequence

FIG. 15B

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/01226

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC: IPC4: C12P 21/00, C12N 1/00; C07H 15/12, A61K 37/00, A61K 37/02		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	435/68, 172.3, 240, 253, 255, 317 536/27; 530/350+ 424/85, 88; 935/9, 10, 32, 62	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
Computer Search CAS, Biosis, Lexis Vaccine for Contraception DNA encoding Beta-HCG, Vaccinia Virus Vector		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	PCT, WO 85/01958 (REDDY) 9 May 1985. See entire document.	7-15
Y	PCT, WO 85/01959 (REDDY) 9 May 1985. See entire document.	7-15
Y	Proc. Natl Acad. Sci. USA (Washington, D.C., USA) Volume 82, issued June 1985 (Reddy et al) "Expression of Human Choriogonadotropin in Monkey Cells Using a Single SV40 Vector". See pages 3644-3648.	1-15
<p>¹⁶ Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ¹	Date of Mailing of this International Search Report ²	
21 August 1986	04 SEP 1986	
International Searching Authority ¹	Signature of Authorized Officer ²⁰	
ISA/US	Stephanie Seidman P.D., J.R. Stephanie Seidman	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	<u>Fertility and Sterility</u> (Philadelphia, Pennsylvania, USA) Volume 36, issued July 1981 (Stevens et al.) "Antifertility Effects of Immunization of Female Baboons with C-Terminal Peptides of the B-Subunit of Human Chorionic Gonadotropin". See pages 98-106.	1-15
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V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 10

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 11

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
Y	<u>Proc. Natl. Acad. Sci. USA</u> (Washington, D.C., USA) Volume 80, issued December 1983 (Smith et al.) "Construction and Characterization of an Infectious Vaccinia Virus Recombinant that Expresses the Influenza Hemagglutinin Gene and Induces Resistance to Influenza Virus Infection in Hamsters". See pages 715-7159.	1-15
Y	<u>Nature</u> (London, England), Volume 302 issued April 1983 (Smith et al.) "Infectious Vaccinia Virus Recombinants that Express Hepatitis B Virus Surface Antigen". See pages 490-495.	1-15
Y	<u>Proc. Natl. Acad. Sci. USA</u> (Washington, D.C. USA) Volume 81 issued September 1984 (Tadeusz et al.) "Protection from Rabies by a vaccinia Virus Recombinant Containing the Rabies Virus Glycoprotein Gene". See pages 7192-7198.	1-15
A	<u>Journal of Andrology</u> (Birmingham, Alabama, USA) Volume 4 issued July 1983 (Schanbacher et al.) "Animal Model of Isolated Gonadotropin Deficiency". See pages 233-239.	1-15
Y	<u>Proc. Natl. Acad. Sci. USA</u> (Washington, D.C. USA) Volume 82 issued February 1985 (Talwar et al.) "Bioeffective Monoclonal Antibody against the Decapeptide Gonadotropin-Releasing Hormone: Reacting Determinant and Action on Ovulation and Estrus Suppression". See pages 1228-1231.	1-15
Y	<u>Proc. Natl. Acad. Sci. USA</u> (Washington D.C., USA) Volume 81 issued August 1984 (Shen et al.) "Multiple Joined Genes Prevent Product Degradation". See pages 4627-4631.	1-6